

**THE UNITED STATES PATENT AND TRADEMARK OFFICE  
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES**

Appellant(s): Bertholet et al.  
Appl. No.: 10/658,522  
Conf. No.: 1947  
Filed: September 8, 2003  
Title: LONG-CHAIN POLYUNSATURATED FATTY ACID OIL AND  
COMPOSITIONS AND PREPARATION PROCESS FOR THE SAME  
Art Unit: 1615  
Examiner: E. Silverman  
Docket No.: 112701-530

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

**APPELLANTS' APPEAL BRIEF**

Sir:

Appellants submit this Appeal Brief in support of the Notice of Appeal filed on September 11, 2007. This Appeal is taken from the Final Rejections in the Office Action dated June 13, 2007.

# **I. REAL PARTY IN INTEREST**

The real party in interest for the above-identified patent application on Appeal is Nestec S.A. by virtue of an Assignment dated January 13, 2004 and recorded at reel 014896, frames 0011-0015 in the United States Patent and Trademark Office.

## **II. RELATED APPEALS AND INTERFERENCES**

Appellants' legal representative and the Assignee of the above-identified patent application do not know of any prior or pending appeals, interferences or judicial proceedings which may be related to, directly affect or be directly affected by or have a bearing on the Board's decision with respect to the above-identified Appeal.

### **III. STATUS OF CLAIMS**

Claims 1-19 are pending in the above-identified patent application. Claims 1-19 stand rejected. Therefore, Claims 1-19 are being appealed in this Brief. A copy of the appealed claims is included in the Claims Appendix.

#### IV. STATUS OF AMENDMENTS

A Non-Final Office Action was mailed on February 2, 2006. In the Non-Final Office Action, the Examiner entered the previous amendments submitted on December 9, 2005 and considered the response but maintained the obviousness rejections and entered new grounds of rejection based on the written description requirement. Appellants filed a response to the Non-Final Office Action on June 1, 2006 with amendments to the claims. A Final Office Action was mailed on July 14, 2006. In the Final Office Action, the Examiner entered the amendments and withdrew the written description rejections but maintained the obviousness rejections. Appellants filed a response to the Final Office Action on October 13, 2006 with amendments to the claims. An Advisory Action was mailed on November 2, 2006. In the Advisory Action, the Examiner did not enter the amendments. Appellants filed a Request for Continued Examination on November 27, 2006. A Non-Final Office Action in response to a Request for Continued Examination was mailed on January 23, 2007. In the Non-Final Office Action, the Examiner entered the previous amendments, but maintained the obviousness rejections under 35 U.S.C. §103. Appellants filed a response to the Non-Final Office Action on April 23, 2007. A Final Office Action was mailed on June 13, 2007. In the Final Office Action, the Examiner maintained the obviousness rejections. In the Final Office Action, the Examiner failed to address the rejections relating to Claims 4, 10 and 11 identified as numerals 3 and 4 and set forth in the Grounds of Rejection to be Reviewed on Appeal herein below. Appellants note, therefore, that the rejections are considered to have been maintained by the Examiner and are addressed by Appellants herein below. Appellants filed a Notice of Appeal on September 11, 2007 with respect to the Final Office Action mailed on June 13, 2007. Copies of the Non-Final Office Action mailed on February 2, 2006, the Non-Final Office Action mailed on January 23, 2007, and the Final Office Action mailed on June 13, 2007 are attached as Exhibits A, B, and C, respectively, in the Evidence Appendix.

## V. SUMMARY OF CLAIMED SUBJECT MATTER

A summary of the invention by way of reference to the specification and/or figures for each of the independent claims is provided as follows:

Independent Claim 1 recites a stable oil for incorporation in a food, nutritional, pharmaceutical or cosmetic product (page 3, lines 10-13, page 7, lines 3-19), the stable oil made by a process comprising combining a carrier oil (page 6, lines 14-23) and one or more long-chain polyunsaturated fatty acids (page 4, lines 32-37) from a biomass (page 3, lines 22-38, page 5, lines 37-38, page 6, lines 1-2) obtained from the culture of a microorganism (page 2, lines 27-29), in the form of triacylglycerols (page 2, lines 21-24), wherein the long-chain polyunsaturated fatty acids are incorporated into the carrier oil such that at least 60% by weight of the long-chain polyunsaturated fatty acids present in the biomass are present in the carrier oil (page 9, lines 5-6), but that less than 10% of phosphorous that is present in the biomass is present in the carrier oil (page 9, lines 7-10), so that the carrier oil does not require purification prior to use (page 4, lines 5-12).

Independent Claim 7 recites a process for preparing a stable oil for incorporation into a composition of a food, nutritional, pharmaceutical or cosmetic product (page 3, lines 10-13, page 7, lines 3-19), the process comprising bringing a carrier oil (page 6, lines 14-23) into contact with a biomass (page 3, lines 22-38, page 5, lines 37-38, page 6, lines 1-2) obtained from the culture of a microorganism (page 2, lines 27-29) containing one or more long-chain polyunsaturated fatty acids (page 4, lines 32-37), so as to transfer the long-chain polyunsaturated fatty acid(s) in the form of triacylglycerols (page 2, lines 21-24) to the carrier oil and form a biomass residue (page 3, lines 13-17), separating the carrier oil containing the fatty acid(s) from the biomass residue (page 3, lines 20-21), and then deodorizing the separated carrier oil to obtain the stable oil (page 4, lines 13-16).

Although specification citations are given in accordance with C.F.R. 1.192(c), these reference numerals and citations are merely examples of where support may be found in the specification for the terms used in this section of the Brief. There is no intention to suggest in any way that the terms of the claims are limited to the examples in the specification. As demonstrated by the references numerals and citations, the claims are fully supported by the specification as required by law. However, it is improper under the law to read limitations from

the specification into the claims. Pointing out specification support for the claim terminology as is done here to comply with rule 1.192(c) does not in any way limit the scope of the claims to those examples from which they find support. Nor does this exercise provide a mechanism for circumventing the law precluding reading limitations into the claims from the specification. In short, the references numerals and specification citations are not to be construed as claim limitations or in any way used to limit the scope of the claims.

## VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Claims 1-3, 5-7, 9, 12-19 are rejected under 35 U.S.C. §103(a) as being unpatentable over U.S. Patent No. 6,177,580 to Timmermann et al. ("*Timmermann*") in view of EP 0726321 A2 to Barclay ("*Barclay*"). Copies of *Timmermann* and *Barclay* are attached herewith as Exhibits D and E, respectively, in the Evidence Appendix.
2. Claims 8-10 are rejected under 35 U.S.C. §103(a) as being unpatentable over *Timmermann* in view of *Barclay* and in further view of U.S. Patent No. 5,773,075 to Todd ("*Todd*"). A copy of *Todd* is attached herewith as Exhibit F in the Evidence Appendix.
3. Claims 10 and 11 are rejected under 35 U.S.C. §103(a) as being unpatentable over *Timmermann* in view of *Barclay* and in further view of U.S. Patent No. 5,840,945 to Tsujiwaki et al. ("*Tsujiwaki*"). A copy of *Tsujiwaki* is attached herewith as Exhibit G in the Evidence Appendix.
4. Claim 4 is rejected under 35 U.S.C. §103(a) as being unpatentable over *Timmermann* in view of *Barclay* and in further view of U.S. Patent No. 5,407,957 to Kyle et al. ("*Kyle*"). A copy of *Kyle* is attached herewith as Exhibit H in the Evidence Appendix.



## VII. ARGUMENT

### A. LEGAL STANDARDS

#### Obviousness under 35 U.S.C. §103

The Federal Circuit has held that the legal determination of an obviousness rejection under 35 U.S.C. § 103 is:

whether the claimed invention as a whole would have been obvious to a person of ordinary skill in the art at the time the invention was made...The foundational facts for the *prima facie* case of obviousness are: (1) the scope and content of the prior art; (2) the difference between the prior art and the claimed invention; and (3) the level of ordinary skill in the art...Moreover, objective indicia such as commercial success and long felt need are relevant to the determination of obviousness...Thus, each obviousness determination rests on its own facts.

*In re Mayne*, 41 U.S.P.Q. 2d 1451, 1453 (Fed. Cir. 1997).

In making this determination, the Patent Office has the initial burden of proving a *prima facie* case of obviousness. *In re Rijckaert*, 9 F.3d 1531, 1532, 28 U.S.P.Q. 2d 1955, 1956 (Fed. Cir. 1993). This burden may only be overcome “by showing some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art would lead that individual to combine the relevant teachings.” *In re Fine*, 837 F.2d 1071, 1074, 5 U.S.P.Q. 2d 1596, 1598 (Fed. Cir. 1988). “If the examination at the initial stage does not produce a *prima facie* case of unpatentability, then without more the applicant is entitled to grant of the patent.” *In re Oetiker*, 24 U.S.P.Q. 2d 1443, 1444 (Fed. Cir. 1992).

Moreover, the Patent Office must provide explicit reasons why the claimed invention is obvious in view of the prior art. The Supreme Court has emphasized that when formulating a rejection under 35 U.S.C. § 103(a) based upon a combination of prior art elements it remains necessary to identify the reason why a person of ordinary skill in the art would have combined the prior art elements in the manner claimed. *KSR v. Teleflex*, 127 S. Ct. 1727 (2007).

Of course, references must be considered as a whole and those portions teaching against or away from the claimed invention must be considered. *Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve Inc.*, 796 F.2d 443 (Fed. Cir. 1986). “A prior art reference may be considered to teach away when a person of ordinary skill, upon reading the reference would be discouraged

from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the Applicant.” *Monarch Knitting Machinery Corp. v. Fukuhara Industrial Trading Co., Ltd.*, 139 F.3d 1009 (Fed. Cir. 1998), quoting, *In re Gurley*, 27 F.3d 551 (Fed. Cir. 1994).

B. THE CLAIMED INVENTION

Independent Claim 1 recites, in part, in part, a stable oil for incorporation in a food, nutritional, pharmaceutical or cosmetic product. The stable oil is made by a process comprising combining a carrier oil and one or more long-chain polyunsaturated fatty acids from a biomass obtained from the culture of a microorganism. The long-chain polyunsaturated fatty acids are in the form of triacylglycerols. The long-chain polyunsaturated fatty acids are incorporated into the carrier oil such that at least 60% by weight of the long-chain polyunsaturated fatty acids present in the biomass are present in the carrier oil but that less than 10% of phosphorous that is present in the biomass is present in the carrier oil. The long-chain polyunsaturated fatty acids are incorporated into the carrier oil such that the carrier oil does not require purification prior to use.

Independent Claim 7 recites, in part, a process for preparing a stable oil for incorporation into a food, nutritional, pharmaceutical or cosmetic product. The process comprises bringing a carrier oil into contact with a biomass obtained from the culture of a microorganism containing one or more long-chain polyunsaturated fatty acids. The carrier oil is brought into contact with the biomass so as to transfer the long-chain polyunsaturated fatty acid(s) in the form of triacylglycerols to the carrier oil and form a biomass residue. The carrier oil containing the fatty acid(s) is separated from the biomass residue. The separated carrier oil is then deodorized to obtain the stable oil.

C. THE REJECTION OF CLAIMS 1-3, 5-7, 9, AND 12-19 UNDER 35 U.S.C. §103(A) TO TIMMERMAN AND BARCLAY SHOULD BE REVERSED BECAUSE THE EXAMINER HAS NOT ESTABLISHED A PRIMA FACIE CASE OF OBVIOUSNESS

Appellants respectfully submit that there exists no reason why the skilled artisan would combine the cited references to arrive at the present claims and, even if combinable, the cited

references fail to disclose or suggest every element of the presently pending claims. Independent Claims 1 and 7 recite, in part, one or more long-chain polyunsaturated fatty acids incorporated into a carrier oil from a biomass obtained from the culture of a microorganism. In contrast, *Timmermann* and *Barclay* fail to suggest or teach every element of independent Claims 1 and 7 for at least the reasons set forth below.

1. There exists no reason why the skilled artisan would combine *Timmermann* and *Barclay* to arrive at the present claims

Independent Claims 1 and 7 require, in part, one or more long-chain polyunsaturated fatty acids incorporated into a carrier oil from a biomass obtained from the culture of a microorganism. *Timmermann* teaches a synthetic chemical process for making triacylglycerols rich in conjugated linoleic acid residues. See, *Timmermann*, column 2, lines 19-41. Conjugated linoleic acid is not a long-chain polyunsaturated fatty acid (LC-PUFA). In fact, as acknowledged by the Examiner, *Timmermann* does not disclose LC-PUFAs and does not teach using microorganisms. See, Non-Final Office Action dated February 2, 2006, page 4, lines 7, 10. *Barclay* teaches a biological process for obtaining arachidonic acid from biomass produced by culturing a specific microorganism. The different approaches taken by the cited references are incompatible and thus teach away from the proposed combination. For at least the reasons set forth below, Appellants respectfully submit that one having ordinary skill in the art would have no reason to modify or combine *Timmermann* and *Barclay* to obtain the present invention.

Appellants respectfully submit that one having ordinary skill in the art would not be motivated to combine *Timmermann* and *Barclay* because such a combination would change the principle of operation of *Barclay* and render such reference inoperable. A proposed modification or combination of prior art that would change the principle of operation of one of the prior art inventions is not sufficient to support a prima facie case of obviousness. *In re Ratti*, 270 F.2d 810, 813 (C.C.P.A. 1959). For example, the only basis cited by the Examiner to combine the cited references is that *Barclay* allegedly teaches that while arachidonic acid can be obtained from certain oils deemed useful by *Timmermann*, such as fish oils, it is preferable to use microorganisms. See, Non-Final Office Action dated February 2, 2006, page 4, lines 12-19. *Timmermann* teaches a required process step of heating the reaction mixture to a temperature of

180-240°C to form the triacylglycerol. See *Timmermann*, column 4, lines 5-14. Appellants respectfully submit that subjecting the microorganisms in *Barclay* to those temperatures would render such microorganisms inoperable for *Barclay*'s intended function of producing arachidonic acid. The technical reasoning or evidence for Appellants' assertion, sought by the Examiner in the Final Office Action dated June 13, 2007, page 3, lines 11-12, can be found in *Barclay* itself. For example, *Barclay* specifically states that "[g]rowth strains of [the microorganism] of the present invention can be effected at any temperature conducive to satisfactory growth of the strain; for example, between about 25°C and 33°C, preferably between about 27°C and 32°C, and more preferably at about 30°C." See, *Barclay*, page 5, lines 37-39. Thus the microorganisms of *Barclay* cannot be grown at a temperature of 180-240°C, a range far outside that disclosed in *Barclay* for effecting growth of the microorganisms. Therefore, Appellants respectfully submit that one of ordinary skill in the art would have no reason to combine *Barclay* with the high-temperature process of *Timmermann* to arrive at the present claims.

Although the Examiner contends that the heating procedure in *Timmermann* could only render the process claims of *Barclay* inoperable, see, Office Action dated June 13, 2007, page 3, lines 9-11, Appellants respectfully submit that the heat requirement of *Timmermann* would render the entire reference of *Barclay* inoperable, including the product claims. The product claims of *Barclay* all require the *Mortierella* sect. *schmuckeri* genus of microorganism disclosed in *Barclay* and products recovered therefrom. See, *Barclay*, page 14, line 57, page 15, lines 1-14, 18-23. Thus, Appellants respectfully submit that all product and process claims in *Barclay* require the microorganism grown or cultured as disclosed in the reference. Because *Barclay* teaches that the *Mortierella* sect. *schmuckeri* microorganism of the invention can only be grown at temperatures of approximately 25-33°C, see, *Barclay*, page 5, lines 37-39, Appellants respectfully submit that one of ordinary skill in the art would have no reason to combine *Timmermann*, which requires a high temperature of 180-240°C, with the microorganisms of *Barclay* to arrive at the present claims.

Moreover, the cited references teach entirely different processes for making their final products. *Timmermann* teaches a chemical process of making synthetic triacylglycerols rich in conjugated linoleic acid residues. See, *Timmermann*, column 2, lines 19-41. As the Examiner admits, *Timmermann* does not refer to any biomass or microorganisms. See, Non-Final Office Action dated February 2, 2006, page 4, lines 7-8. *Timmermann* requires heating a reaction

mixture to 180-240°C to produce triacylglycerols. See, *Timmermann*, column 4, lines 5-14. *Barclay* teaches a biological process for obtaining arachidonic acid from biomass produced by culturing a microorganism. See, *Barclay*, page 3, lines 9-12. Specifically, *Barclay* teaches culturing a microorganism in a fermentation medium at temperatures of 25-33°C to produce arachidonic acid. See, *Barclay*, page 4, lines 58-59, page 5, lines 37-39. One having ordinary skill in the art would understand that the chemical process of *Timmermann* cannot be applied to the biological process of *Barclay* or vice versa.

In sum, the Examiner has failed to consider the cited references as a whole including those portions teaching against or away from each other and/or the claimed invention. *Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve Inc.*, 796 F.2d 443, 448-49 (Fed. Cir. 1986). “A prior art reference may be considered to teach away when a person of ordinary skill, upon reading the reference would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the [Appellant].” *Monarch Knitting Machinery Corp. v. Fukuhara Industrial Trading Co., Ltd.*, 139 F.3d 1009, (Fed. Cir. 1998). Because heating the microorganisms of *Barclay* in accordance with *Timmermann* would render such microorganisms inoperable and would therefore not produce arachidonic acid according to *Barclay*, one having ordinary skill in the art would be discouraged from applying the process of *Timmermann* to the microorganisms of *Barclay* to obtain a high yield of LC-PUFA as required, in part, by the present invention. Therefore, *Barclay* teaches away from *Timmermann* and one of ordinary skill in the art would have no reason to combine the cited references to obtain the invention of the present claims.

2. Even if combinable, *Timmermann* and *Barclay* do not teach or suggest all of the claimed elements of the present invention

Appellants respectfully submit that, even if combinable, the cited references fail to teach or suggest all of the required elements of the present claims. For example, the cited references fail to disclose or suggest that the LC-PUFAs are incorporated such that at least 60% by weight of the LC-PUFAs present in the biomass are present in the carrier oil but that less than 10% by weight of phosphorous that is present in the biomass is present in the carrier oil as required, in part, by independent Claim 1. *Timmermann* only refers to high yields once in the specification:

“[i]t has also been found that, surprisingly, the triglycerides according to the invention can also readily be obtained in high yields.” See, *Timmermann*, column 2, lines 65-67. *Timmermann* never mentions a specific percentage yield of triglycerides. Contrary to the Examiner’s assertion, *Timmermann* does not disclose or even suggest that the yield should be close to 100%. See, Final Office Action dated June 13, 2007, page 3, lines 21-23. The only percentages of triglycerides disclosed in *Timmermann* are the percent compositions of product containing such triglycerides. See, *Timmermann*, column 3, lines 17-26, column 5, lines 27-34. However, the cited reference never discloses the percent of total triglyceride obtained that is present in the product. For example, the percent composition of a product could be 100% triglyceride but the product may only contain a 5% yield of triglycerides. The Examiner does not provide any support in the reference itself for the assertion that “*Timmermann*’s teaching of high yield is deemed to meet this requirement [of 60% or greater yield] or at a minimum, suggest that the yield should ideally be close to 100% thus suggesting this requirement.” See, Final Office Action dated June 13, 2007, page 3, lines 21-23. Moreover, since *Timmermann* does not suggest or disclose obtaining triglycerides from a biomass obtained by culturing a microorganism, see, Non-Final Office Action dated February 2, 2006, page 4, line 10, Appellants respectfully submit that *Timmermann*’s discussions of yield would not suggest to one of ordinary skill in the art that 60% or greater of triglycerides present in a biomass can be obtained in a carrier oil.

Moreover, even if combinable, *Timmermann* and *Barclay* fail to disclose or suggest a process or product in which less than 10% of the phosphorous present in a biomass is present in the stable oil product simultaneously with 60% of the LC-PUFAs present in the biomass as required, in part, by Claim 1 of the present invention. The Examiner’s only support for the contention that the cited references meet this requirement is that *Timmermann* discloses a high yield of triglycerides and no phospholipids are reported in the product. See, Non-Final Office Action dated February 2, 2006, page 4, lines 5-7. However, since *Timmermann* does not disclose or report any biomass, see, Non-Final Office Action dated February 2, 2006, page 4, line 10, the reference could not report any phosphorous present in the biomass that is obtained in the product. Appellants respectfully submit that the reason *Timmermann* did not discuss or disclose the presence of any phosphorous in the final triglyceride product is that the reference did not disclose using biomass to obtain LC-PUFAs and thus the inventors were not concerned with phosphorous from the biomass being present in the final product.

Finally, the cited references fail to suggest or disclose extracting LC-PUFAs from a biomass simply by use of a carrier oil without further extraction of the oil prior to use as required, in part, by independent Claims 1 and 7 of the present invention. The Examiner acknowledges that *Timmermann* does not disclose or refer to any LC-PUFAs or biomass. See, Non-Final Office Action dated February 2, 2006, page 4, lines 7, 10. Further, Appellants respectfully submit that *Barclay* achieves its objective by using conventional methods of extraction (methods known in the art), such as extraction with solvents or supercritical fluid extraction, because the use of edible oils to extract another oil from a biomass was not a standard method known in the art at that time. In fact, *Barclay* itself refers to recovering the lipids by conventional extraction methods and converting them subsequently into the form of edible oil in another purification step. See, *Barclay*, page 6, lines 27-33. In contrast, the present invention discloses using a carrier oil directly to selectively displace the biomass oil (not unwanted impurities) from the milled biomass. This enables separation of the oil from the biomass residue, for example, by squeezing the resulting biomass-oil slurry in a press. As a consequence, although the pressed cake still retains some oil, it has a very low content of LC-PUFAs because most of the LC-PUFAs have been transferred to the carrier oil. The stable oil obtained by the present invention is clean and does not need to be subjected to further purification. Thus, *Barclay* does not disclose using a carrier oil to extract LC-PUFAs from a biomass as required, in part, by Claims 1 and 7 of the present invention, and the combination of the cited references would not suggest this method to one of ordinary skill in the art.

In sum, the combination of *Timmermann* and *Barclay* fails to disclose each and every element of the present invention. Instead of teaching ways to extract a high percentage of LC-PUFAs from a biomass using a carrier oil without further purification, the cited references only teach ways to obtain LC-PUFAs from a biomass using conventional extraction methods that require further purification before use. Moreover, the cited references do not teach a stable oil containing at least 60% of LC-PUFAs present in the biomass with less than 10% of phosphorous present in the biomass. Thus, the cited references fail to disclose each and every element of the present claims.

D. THE REJECTION OF CLAIMS 8-10 UNDER 35 U.S.C. §103(A) TO *TIMMERMANN, BARCLAY, AND TODD* IS IMPROPER IN VIEW OF THE PATENTABILITY OF INDEPENDENT CLAIM 7

Claims 8-10 are rejected under 35 U.S.C. §103(a) as being unpatentable over *Timmermann, Barclay* and *Todd*. Appellants respectfully submit that the patentability of Claim 7 over *Timmermann* and *Barclay* as discussed previously renders moot the obviousness rejection of Claims 8-10 that depend from Claim 7. In this regard, the cited art fails to teach or suggest the elements of Claims 8-10 in combination with the novel elements of Claim 7.

For example, the Examiner asserts that *Todd* teaches grinding a plant before extraction by an edible oil to increase bioavailability of the desired material and avoid coarse particles in the residual solid cake, and thus it would be obvious to a person of ordinary skill in the art to grind the biomass of the present invention before extraction by a carrier oil. See, Office Action dated February 2, 2006, page 5, lines 6-12. However, one of ordinary skill in the art would not be motivated to combine *Timmermann* and *Barclay* to extract LC-PUFAs from a biomass using a carrier oil for the reasons discussed above. Thus, *Todd* does not cure the deficiencies of *Timmermann* and *Barclay*.

Accordingly, Appellants respectfully submit that Claims 8-10 are novel, nonobvious and distinguishable from the cited references and are in condition for allowance.

E. THE REJECTION OF CLAIMS 10 AND 11 UNDER 35 U.S.C. §103(A) TO *TIMMERMANN, BARCLAY, AND TSUJIWAKI* IS IMPROPER IN VIEW OF THE PATENTABILITY OF INDEPENDENT CLAIM 7

Claims 10 and 11 are rejected under 35 U.S.C. §103(a) as being unpatentable over *Timmermann, Barclay* and *Tsujiwaki*. Appellants respectfully submit that the patentability of Claim 7 over *Timmermann* and *Barclay* as discussed previously renders moot the obviousness rejection of Claims 10 and 11 that depend from Claim 7. In this regard, the cited art fails to teach or suggest the elements of Claims 10 and 11 in combination with the novel elements of Claim 7.



For example, the Examiner alleges that “*Tsujiwaki* teaches that PUFAs are susceptible to oxidation in air, and thus it is common in the art to add tocopherols to PUFAs as antioxidants.” See, Non-Final Office Action dated February 2, 2006, page 5, lines 19-20. However, as discussed previously, one of ordinary skill in the art would not be motivated to combine *Timmermann* and *Barclay* and, even if combinable, the combination of cited references does not disclose combining a carrier oil with a biomass to extract LC-PUFAs and obtain a stable oil that does not require purification prior to use, as required, in part, by Claim 7. Therefore, *Tsujiwaki* does not cure the deficiencies of *Timmermann* and *Barclay* and does not teach or suggest the required elements of Claims 10 and 11 that depend from independent Claim 7.

Accordingly, Appellants respectfully submit that Claims 10 and 11 are novel, nonobvious and distinguishable from the cited references and are in condition for allowance.

F. THE REJECTION OF CLAIM 4 UNDER 35 U.S.C. §103(A) TO *TIMMERMANN*, *BARCLAY*, AND *KYLE* IS IMPROPER IN VIEW OF THE PATENTABILITY OF INDEPENDENT CLAIM 1

Claim 4 is rejected under 35 U.S.C. §103(a) as being unpatentable over *Timmermann*, *Barclay* and *Kyle*. Appellants respectfully submit that the patentability of Claim 1 over *Timmermann* and *Barclay* as discussed previously renders moot the obviousness rejection of Claim 4 that depends from Claim 1. In this regard, the cited art fails to teach or suggest the elements of Claim 4 in combination with the novel elements of Claim 1.

For example, Claim 4 requires that docosahexanoic acid (DHA) be the stable oil. The Examiner asserts that *Kyle* teaches that docosahexanoic acid (DHA) is useful in foods, such as infant products. See, Non-Final Office Action dated January 23, 2007, page 5, lines 19-20. Because the arachidonic acid taught in *Barclay* may also be used in infant products, the Examiner asserts it would be obvious to one of ordinary skill in the art to use the DHA of *Kyle* in place of the arachidonic acid of *Barclay* to arrive at the product of Claim 4. See, Office Action dated January 23, 2007, page 6, lines 3-5. However, for reasons discussed above, one of ordinary skill in the art would have no reason to combine *Timmermann* and *Barclay* to arrive at

the present invention and, even if combinable, the cited references do not disclose or suggest every element of the present Claim 1.

Accordingly, Appellants respectfully submit that Claim 4 is novel, nonobvious and distinguishable from the cited references and is in condition for allowance.

For at least the reasons discussed above, there exists no reason why the skilled artisan would combine the cited references of *Timmermann* and *Barclay*. Furthermore, even if combinable, the cited references fail to disclose or suggest every element of the present claims. Accordingly, Appellants respectfully submit that independent Claims 1 and 7 and Claims 2-6, 8-19 that depend from Claims 1 and 7 are novel, nonobvious and distinguishable from the cited references and are in condition for allowance.

### VIII. CONCLUSION


Appellants respectfully submit that the Examiner has failed to establish obviousness under 35 U.S.C. §103(a) with respect to the rejections of Claims 1-19. Accordingly, Appellants respectfully submit that the obviousness rejections are erroneous in law and in fact and should therefore be reversed by this Board.

The Director is authorized to charge \$500 for the Appeal Brief and any additional fees which may be required, or to credit any overpayment to Deposit Account No. 02-1818. If such a withdrawal is made, please indicate the Attorney Docket No. 112701-530 on the account statement.

Respectfully submitted,

BELL, BOYD & LLOYD LLP

BY

  
\_\_\_\_\_  
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Dated: November 9, 2007

## CLAIMS APPENDIX

### PENDING CLAIMS ON APPEAL OF U.S. PATENT APPLICATION SERIAL NO. 10/658,522

1. A stable oil for incorporation in a food, nutritional, pharmaceutical or cosmetic product, the stable oil made by a process comprising combining a carrier oil and one or more long-chain polyunsaturated fatty acids from a biomass obtained from the culture of a microorganism, in the form of triacylglycerols, wherein the long-chain polyunsaturated fatty acids are incorporated into the carrier oil such that at least 60% by weight of the long-chain polyunsaturated fatty acids present in the biomass are present in the carrier oil but that less than 10% of phosphorous that is present in the biomass is present in the carrier oil, so that the carrier oil does not require purification prior to use.

2. The stable oil according to claim 1, in which the long-chain polyunsaturated fatty acid is arachidonic acid, dihomogammalinolenic acid, eicosapentaenoic acid or docosahexaenoic acid.

3. The stable oil according to claim 2, in which the long-chain polyunsaturated fatty acid is arachidonic acid.

4. The stable oil according to claim 2, in which the long-chain polyunsaturated fatty acid is docosahexaenoic acid.

5. The stable oil according to claim 1, in which the carrier oil is high oleic acid sunflower oil (HOSFO), sunflower oil (SFO), soya bean oil, palm olein or a medium-chain triacylglycerol (MCT, containing essentially triacylglycerols of saturated C<sub>8</sub>-C<sub>10</sub> fatty acids).

6. The stable oil according to claim 1, wherein the stable oil contains no more than 10% by weight of LC-PUFAs.

7. A process for preparing a stable oil for incorporation into a composition of a food, nutritional, pharmaceutical or cosmetic product, the process comprising bringing a carrier oil into contact with a biomass obtained from the culture of a microorganism containing one or more long-chain polyunsaturated fatty acids, so as to transfer the long-chain polyunsaturated fatty acid(s) in the form of triacylglycerols to the carrier oil and form a biomass residue, separating the carrier oil containing the fatty acid(s) from the biomass residue, and then deodorizing the separated carrier oil to obtain the stable oil.

8. The process according to claim 7, which further comprises grinding the biomass prior to contact with the carrier oil to reduce particle size in order to break cell walls of the microorganisms and to thereby increase contact surface area between the carrier oil and the biomass.

9. The process according to claim 7, which further comprises contacting the biomass under high pressure with the carrier oil, and separating the oil containing the long-chain polyunsaturated fatty acid from the biomass residue by pressing and filtration.

10. The process according to claim 7, which further comprises grinding the biomass in the presence of the carrier oil under an inert atmosphere.

11. The process according to claim 7, which is carried out under a nitrogen atmosphere and in the presence of one or more tocopherols in an amount sufficient to protect the LC-PUFAs from oxidative degradation.

12. The process according to claim 7, wherein the deodorizing is achieved by molecular distillation or steam distillation, optionally assisted by vacuum.

13. The process according to claim 7, which further comprises a fine filtration of the stable oil to remove fine biomass particles.

14. The stable oil obtained by the process of claim 7.

15. A product containing a stable oil according to claim 14, wherein the stable oil contains no more than 10% by weight of LC-PUFAs.

16. The product of claim 15 wherein the product is an infant foodstuff, a nutritional composition, a cosmetic composition, or a foodstuff.

17. A product containing a stable oil according to claim 1, wherein the stable oil contains no more than 10% by weight of LC-PUFAs.

18. The product of claim 17 wherein the product is an infant foodstuff, a nutritional composition, a cosmetic composition, or a foodstuff.

19. An animal feed containing the biomass residue obtained by the process according to claim 7.

## EVIDENCE APPENDIX

EXHIBIT A: Non-Final Office Action dated February 2, 2006

EXHIBIT B: Non-Final Office Action dated January 23, 2007

EXHIBIT C: Final Office Action dated June 13, 2007

EXHIBIT D: United States Patent No. 6,177,580 to Timmermann et al. ("*Timmermann*"), cited by the Examiner in the Non-Final Office Actions dated February 2, 2006 and January 23, 2007 and the Final Office Action dated June 13, 2007

EXHIBIT E: EP 0726321 A2 to Barclay ("*Barclay*"), cited by the Examiner in the Non-Final Office Actions dated February 2, 2006 and January 23, 2007 and the Final Office Action dated June 13, 2007

EXHIBIT F: United States Patent No. 5,773,075 to Todd ("*Todd*"), cited by the Examiner in the Non-Final Office Actions dated February 2, 2006 and January 23, 2007 and the Final Office Action dated June 13, 2007

EXHIBIT G: United States Patent No. 5,840,945 to Tsujiwaki et al. ("*Tsujiwaki*"), cited by the Examiner in the Non-Final Office Actions dated February 2, 2006 and January 23, 2007

EXHIBIT H: United States Patent No. 5,407,957 to Kyle et al. ("*Kyle*"), cited by the Examiner in the Non-Final Office Action dated January 23, 2007

# EXHIBIT A





# UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/658,522	09/08/2003	Raymond Bertholet	88265-6925	1947
29157	7590	02/02/2006		
BELL, BOYD & LLOYD LLC P. O. BOX 1135 CHICAGO, IL 60690-1135			EXAMINER SILVERMAN, ERIC E	
			ART UNIT 1615	PAPER NUMBER

DATE MAILED: 02/02/2006

*Due: 5-2-06*

References Downloaded

Please find below and/or attached an Office communication concerning this application or proceeding.

**RECEIVED**  
BELL, BOYD & LLOYD  
INTELLECTUAL PROPERTY DOCKET  
FEB 06 2006  
ATTY *hmb-nwb*  
DOCKET # *1127017530*

# Office Action Summary

Application No.

10/658,522

Applicant(s)

BERTHOLET ET AL.

Examiner

Eric E. Silverman, PhD

Art Unit

1615

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

## Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

## Status

- 1) ☐ Responsive to communication(s) filed on \_\_\_\_.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

## Disposition of Claims

- 4) ☒ Claim(s) 1-19 is/are pending in the application.
- 4a) Of the above claim(s) \_\_\_\_ is/are withdrawn from consideration.
- 5) ☐ Claim(s) \_\_\_\_ is/are allowed.
- 6) ☒ Claim(s) 1-19 is/are rejected.
- 7) ☐ Claim(s) \_\_\_\_ is/are objected to.
- 8) ☐ Claim(s) \_\_\_\_ are subject to restriction and/or election requirement.

## Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on \_\_\_\_ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.  
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).  
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

## Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some \* c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
- \* See the attached detailed Office action for a list of the certified copies not received.

## Attachment(s)

- 1) ☒ Notice of References Cited (PTO-892)
- 2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
- 3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08)  
Paper No(s)/Mail Date \_\_\_\_.
- 4) ☐ Interview Summary (PTO-413)  
Paper No(s)/Mail Date. \_\_\_\_.
- 5) ☐ Notice of Informal Patent Application (PTO-152)
- 6) ☐ Other: \_\_\_\_.

### DETAILED ACTION

Receipt of Amendment and remarks filed therewith, filed 1/05/2006 is acknowledged. Claims 1 – 19 are pending in this action.

#### *Response to Arguments*

Applicant's arguments with respect to the rejection of claims 1 – 19 under 35 U.S.C. 102(b) and 35 U.S.C. 103(a) have been considered but are moot in view of the new ground(s) of rejection detailed below.

#### ***Claim Rejections - 35 USC § 112***

The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1 – 6 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. **This is a written description rejection.**

Claim 1 recites "obtained from the culture of a microorganism." The specification does not describe how to obtain the claimed product from the culture of a microorganism, or what microorganism could be used to obtain the biomass. In fact, all of the examples merely recite "a biomass", which generic to biomasses obtained from cultures of microorganisms and other biomasses.

Claims 2 – 6 and 17 – 19 are rejected for depending on claim 1, while not rectifying the inadequate description thereof.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

In light of amendment, the rejection of claims 10, 12 and 13 under the second paragraph of 35 U.S.C. 112 is withdrawn.

Claims 7 – 16, 18 and 19 **remain** rejected under 35 U.S.C. 112, second paragraph, as being indefinite for reasons of record.

### ***Response to Arguments***

Applicants arguments have been fully considered, but are not persuasive. Regardless of what applicant may intend by the limitation “without purification”, the steps recited in instant claims, such as centerfugation, distillation, and deodorizing are purification steps, since they separate unwanted materials from desired materials.

### ***Claim Rejections - 35 USC § 103***

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Claims 1 – 3, 5 – 7, 9, 12, 13, 14 – 19 are rejected under 35 U.S.C. 103(a) as being unpatentable over Timmermann et al. US 6,177,580, in combination with Barclay, EP 0726321, of record.

Timmermann teaches the synthetic triglycerols and methods of manufacture thereof (abstract). The triglycerols are manufactured by transesterification of fatty acid glycerides, such as fish oil, with edible oils, such as palm kernel or coco (col. 3, line 49 – col. 4, line 21). The process is carried out under inert gas, and in high yield (col. 2, lines 46 – 64). High yield is deemed to be a teaching of more than 60%, as required by instant claim 1. No phospholipids are reported in the product, so such are deemed absent. No LC-PUFAs are reported in the product, so such are also deemed absent. Also, the steps of distillation and filtering are taught (Examples 1 – 3). compounds, especially in infant diets (page 2, lines 10 – 21).

Timmermann does not teach using microorganisms.

Barclay teaches that PUFA's in general and arachadonic acid in specific are important dietary. Barclay also teaches that while arachadonic acid can be obtained from fish oils, it is present in a low concentration there, and thus it is preferably to use microorganisms (page 2, lines 28 – 50).

Accordingly, it would be prime facie obvious to a person of ordinary skill in the art at the time of the invention to apply the product and method of Timmermann to microorganisms, as taught by Barclay. The motivation to do this comes from Barclay, who teaches that certain oils deemed useful by Timmermann, such as fish oils, have a lower concentration of beneficial oil than do microorganisms. Accordingly the artisan would enjoy a reasonable expectation of success.

Claims 9 and 10 are rejected under 35 U.S.C. 103(a) as being unpatentable over Timmermann et al. US 6,177,580, in combination with Barclay, EP 0726321, of record

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as applied to claims 1 – 3, 5 – 7, 9, 12, 13, 14 – 19 above, and further in view of Todd, of record.

The teachings of Timmermann and Barclay are discussed above.

Timmermann and Barclay do not teach grinding the biomass before contacting it with the oil.

Todd teaches grinding a plant before extraction by an edible oil to increase the bioavailability of the desired material and to avoid coarse particles in the residual solid cake (paragraph bridging col.'s 5 – 6).

Accordingly, a person of ordinary skill in the art would be motivated to grind the biomass, as taught by Todd, in order to increase the bioavailability and to avoid coarse particles in the solid cake. Since Todd teaches this in an extraction process, the artisan would have a reasonable expectation of success.

Claim 10 is rejected under 35 U.S.C. 103(a) as being unpatentable over Timmermann et al. US 6,177,580, in combination with Barclay, EP 0726321, of record as applied to claims 1 – 3, 5 – 7, 9, 12, 13, 14 – 19 above, and further in view of Tsujiwaki, of record.

The teachings of Timmermann and Barclay are discussed above.

Timmermann and Barclay do not teach the use of tocopherols.

Tsujiwaki teaches that PUFA's are susceptible to oxidation in air, and thus it is common in the art to add tocopherols to PUFA's as antioxidants.

Accordingly, it would be prime facie obvious to a person of ordinary skill in the art at the time of the invention to add tocopherols to the PUFA's of Timmermann and

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Barclay. The motivation is provided by Tsujiwaki, who leads the artisan to believe that the PUFA compositions will be more stable if tocopherols are added. Thus, the artisan would enjoy a reasonable expectation of success.

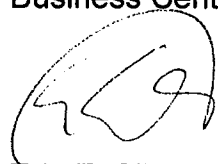
### ***Conclusion***

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Eric E. Silverman, PhD whose telephone number is 571 272 5549. The examiner can normally be reached on Monday to Friday 7:30 am to 4:00 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Thurman K. Page can be reached on 571 272 0602. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).



Eric E. Silverman, PhD  
Art Unit 1615



Gollamudi S. Kishore, PhD  
Primary Examiner  
Group 1600

<b>Notice of References Cited</b>	Application/Control No. 10/658,522	Applicant(s)/Patent Under Reexamination BERTHOLET ET AL.	
	Examiner Eric E. Silverman, PhD	Art Unit 1615	Page 1 of 1

**U.S. PATENT DOCUMENTS**

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-6,177,580	01-2001	Timmermann et al.	554/169
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

**FOREIGN PATENT DOCUMENTS**

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

**NON-PATENT DOCUMENTS**

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

\*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)  
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.



# **EXHIBIT B**



UNITED STATES DEPARTMENT OF COMMERCE  
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/658,522	09/08/2003	Raymond Bertholet	88265-6925	1947

29157 7590 01/23/2007  
BELL, BOYD & LLOYD LLP  
P.O. Box 1135  
CHICAGO, IL 60690

EXAMINER
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SILVERMAN, ERIC E

ART UNIT	PAPER NUMBER
----------	--------------

1615

*Due: 4/23/07*

SHORTENED STATUTORY PERIOD OF RESPONSE	MAIL DATE	DELIVERY MODE
3 MONTHS	01/23/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

If NO period for reply is specified above, the maximum statutory period will apply and will expire 6 MONTHS from the mailing date of this communication.

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INTELLECTUAL PROPERTY DOCKET  
JAN 29 2007  
ATTY: RMB/MLB  
DOCKET: 112701-530

## Office Action Summary

Application No.

10/658,522

Applicant(s)

BERTHOLET ET AL.

Examiner

Eric E. Silverman, PhD

Art Unit

1615

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

### Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

### Status

- 1) ☒ Responsive to communication(s) filed on 27 November 2006.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

### Disposition of Claims

- 4) ☒ Claim(s) 1-19 is/are pending in the application.
- 4a) Of the above claim(s) \_\_\_\_\_ is/are withdrawn from consideration.
- 5) ☐ Claim(s) \_\_\_\_\_ is/are allowed.
- 6) ☒ Claim(s) 1-19 is/are rejected.
- 7) ☐ Claim(s) \_\_\_\_\_ is/are objected to.
- 8) ☐ Claim(s) \_\_\_\_\_ are subject to restriction and/or election requirement.

### Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on \_\_\_\_\_ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.  
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).  
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

### Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some \* c) ☐ None of:
- ☐ Certified copies of the priority documents have been received.
  - ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_\_.
  - ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

\* See the attached detailed Office action for a list of the certified copies not received.

### Attachment(s)

- |  |   |
|--|---|
| 1) <input type="checkbox"/> Notice of References Cited (PTO-892)   | 4) <input type="checkbox"/> Interview Summary (PTO-413)<br>Paper No(s)/Mail Date. _____ |
| 2) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948)                       | 5) <input type="checkbox"/> Notice of Informal Patent Application                       |
| 3) <input type="checkbox"/> Information Disclosure Statement(s) (PTO/SB/08)<br>Paper No(s)/Mail Date _____ | 6) <input type="checkbox"/> Other: _____  |

### DETAILED ACTION

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submissions filed on 10/13/2006 and 11/27/2006 have been entered.

Claims 1 – 19 are pending.

### ***Double Patenting***

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 1 – 19 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1 - 17 of

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copending Application No. 10/526,576. Although the conflicting claims are not identical, they are not patentably distinct from each other because while copending application requires the process step of mressing the biomasses in the dry state, this is not required by instant claims. However, since instant claims use open "comprising" language, copending claims are merely a species of instant genus claims, thus rendering instant claims obvious in their entirety.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

### ***Claim Rejections - 35 USC § 103***

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Claims 1 – 3, 5 – 7, 9, 12, 13, 14 – 19 **remain** rejected under 35 U.S.C. 103(a) as being unpatentable over Timmermann, US 6,177,580 in combination with Barclay, EP 0726321 for reasons of record and those discussed below.

### ***Response to Arguments***

Applicants' arguments have been fully considered, but are not persuasive.

Applicants' argue that the references are directed to different arts, since they teach different processes for making their final products. However, both references are directed to making dietary lipids useful in foods, infant formulas, and the like, and thus are analogous art. That they take different approaches to solve this problem is not germane.

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Applicants further submit that the Timmermann references teaching of heating would render the microorganisms in Barclay unsuitable to perform its intended function. This argument is only applicable to process claims 7 – 13, since the product of the remaining claims need not be made by the process that Applicants' aver would be impossible to carry out. In response, there is no evidence of record to support Applicants' assertion. Arguments cannot take the place of evidence of record.

Applicants also argue that the references do not teach the incorporation of PUFAs such that at least 60% of the long chain PUFAs in the biomass are present in the carrier oil but that less than 10% of the phosphorous is so present. This is not persuasive, because the teaching of high yield in Timmermann is understood to mean greater than 60%, and also because no phosphorous is noted in any of the products when they are analyzed, thus, such is understood to be absent.

Applicants also argue that in the present invention, and contrary to the art, the carrier oil is used to selectively displace the biomass oil from a milled biomass. This limitation is not present in the claims, and thus is not a basis for patentability.

Claims 9 and 10 **remain** rejected under 35 U.S.C. 103(a) as being unpatentable over Timmermann, Barclay and Todd. Upon consideration, claim 8 is not included in this rejection.

Claim 8 includes the additional step of grinding the biomass. Todd teaches that grinding biomasses before extraction by an edible oil is desired because coarse particles in the residual solid cake can thus be avoided.

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Thus, it would be prime facie obvious to a person of ordinary skill in the art at the time of the invention to grind the biomass of Timmermann and Barclay as taught by Todd. The motivation is to avoid solid particles in the cake.

***Response to Arguments***

Applicants' arguments have been fully considered, but are not persuasive.

Applicants' argue only that Todd does not rectify the alleged deficiencies of Timmermann and Barclay. These supposed deficiencies have been addressed, *supra*.

Claim 10 **remains** rejected under 35 U.S.C. 103(a) as being unpatentable over Timmermann, Barclay, and Tsujiwaki for reasons of record and those discussed below. Upon consideration, claim 11 is also included in this rejection for reasons of record.

***Response to Arguments***

Applicants' have not addressed this rejection in their response. It must therefore be maintained.

Claim 4 is rejected under 35 U.S.C. 103(a) as being unpatentable over Timmermann, Barclay and Kyle, US 5,407,957.

The claim specifies that DHA be the stable oil.

The teachings of Timmermann and Barclay are discussed above.

What is lacking is a teaching of hocosahexanoic acid (DHA).

Kyle teaches that DHA is useful in foods, such as infant formula (col. 1 line 54 – col. 2 line 5). This is noted to be the same use as noted by the art of record for arachadonic acid. Kyle teaches a method of preparing this oil from a biomass, which is

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quite similar to the method used by Barclay to prepare arachadonic acid except that a different organism is used (col. 2 – 3).

It would be prime facie obvious to a person of ordinary skill in the art at the time of the invention to use the DHA of Kyle in place of or in addition to the arachadonic acid of Barclay, thus giving the product of instant claim 4. The motivation is that these nutrients are both recognized in the art as food additives for the same purpose and to the same types of food products (such as infant formula).

### ***Conclusion***


No claims are allowed. No claims are free of the prior art.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Eric E. Silverman, PhD whose telephone number is 571 272 5549. The examiner can normally be reached on Monday to Friday 7:30 am to 4:00 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Michael Woodward can be reached on 571 272 8373. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Eric E. Silverman, PhD  
Art Unit 1615

  
MICHAEL P. WOODWARD  
SUPERVISOR EXAMINER  
TECHNOLOGY CENTER 1600



# EXHIBIT C



# UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/658,522	09/08/2003	Raymond Bertholet	88265-6925	1947

29157 7590 06/13/2007  
BELL, BOYD & LLOYD LLP  
P.O. Box 1135  
CHICAGO, IL 60690

EXAMINER  
SILVERMAN, ERIC E

ART UNIT PAPER NUMBER  
1615

NOTIFICATION DATE DELIVERY MODE  
06/13/2007 ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATENTS@BELLBOYD.COM

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BELL, BOYD & LLOYD  
INTELLECTUAL PROPERTY DOCKET

JUN 19 2007  
ATTY: *[Signature]*  
DOCKET #: *1270*

*530*

**Office Action Summary**

Application No.

10/658,522

Applicant(s)

BERTHOLET ET AL.

Examiner

Eric E. Silverman, PhD

Art Unit

1615

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --  
Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.138(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

- 1) ☒ Responsive to communication(s) filed on 23 April 2007 and 02 May 2007.
- 2a) ☒ This action is FINAL. 2b) ☐ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims**

- 4) ☒ Claim(s) 1-19 is/are pending in the application.
- 4a) Of the above claim(s) \_\_\_\_\_ is/are withdrawn from consideration.
- 5) ☐ Claim(s) \_\_\_\_\_ is/are allowed.
- 6) ☒ Claim(s) 1-19 is/are rejected.
- 7) ☐ Claim(s) \_\_\_\_\_ is/are objected to.
- 8) ☐ Claim(s) \_\_\_\_\_ are subject to restriction and/or election requirement.

**Application Papers**

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on \_\_\_\_\_ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.  
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).  
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

**Priority under 35 U.S.C. § 119**

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some \* c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
  2. ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_\_.
  3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

\* See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

- |  |   |
|--|---|
| 1) <input type="checkbox"/> Notice of References Cited (PTO-892)   | 4) <input type="checkbox"/> Interview Summary (PTO-413)<br>Paper No(s)/Mail Date. _____ |
| 2) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948)                       | 5) <input type="checkbox"/> Notice of Informal Patent Application                       |
| 3) <input type="checkbox"/> Information Disclosure Statement(s) (PTO/SB/08)<br>Paper No(s)/Mail Date _____ | 6) <input type="checkbox"/> Other: _____  |

### DETAILED ACTION

Applicants' submission, filed 5/2/2007, has been received. Claims 1 – 19 are pending.

#### ***Double Patenting***

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

The provisional rejection of claims 1 – 19 as provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1 - 17 of copending Application No. 10/526,576 is **withdrawn as moot** in view of the timely filed terminal disclaimer.

#### ***Claim Rejections - 35 USC § 103***

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and

Art Unit: 1615

the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 1 – 3, 5 – 7 remain rejected under 35 U.S.C. 103(a) as being unpatentable over Timmermann, US 6,177,580 in combination with Barclay EP 0726321 for reasons of record and those discussed below.

### ***Response to Arguments***

Applicants' arguments have been fully considered, but are not persuasive.

Applicant argues that the following the procedures of Timmermann would make the process of Barclay unworkable. This argument is understood to apply only to the instant process claims, and not to the product claims (which do not require and are not limited by process steps). The argument is not persuasive because it is not based on any technical reasoning or evidence. Applicants' merely allege that the temperatures required by Timmermann would render the biological materials of Barclay unsuitable for use, but provide no reasoning or evidence other than their own allegation. Applicants' also argue that the references are not combinable because Barclay teaches biological methods whereas Timmermann is directed to synthetic methods. However, Barclay gives reasons why biological methods are preferable to synthetic methods. As such, the artisan would find it obvious to take the beneficial teachings of Timmermann (high yields, lack of phospholipids and LC-PUFA's), and apply them to the improvements offered by Barclay. Applicants' further argue that the art does not teach 60% or greater. In response Timmermann's teaching of high yield is deemed to meet this requirement, or at a minimum, suggest that the yield should ideally be close to 100% thus suggesting this requirement.

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Claims 8, 9 and 10 remain rejected under 35 U.S.C. 103(a) as being unpatentable over Timmermann, Barclay and Todd.

***Response to Arguments***

Applicants' arguments have been fully considered, but are not persuasive.

Applicants' argue that Todd does not make up for the supposed deficiencies in Timmermann and Barclay. These alleged deficiencies have been addressed, *supra*.

***Conclusion***


**THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire **THREE MONTHS** from the mailing date of this action. In the event a first reply is filed within **TWO MONTHS** of the mailing date of this final action and the advisory action is not mailed until after the end of the **THREE-MONTH** shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than **SIX MONTHS** from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Eric E. Silverman, PhD whose telephone number is 571 272 5549. The examiner can normally be reached on Monday to Friday 7:30 am to 4:00 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Michael Woodward can be reached on 571 272 8373. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

  
MICHAEL P. WOODWARD  
SUPERVISORY PATENT EXAMINER  
TECHNOLOGY CENTER 1600

# **EXHIBIT D**

(12) **United States Patent**  
**Timmermann et al.**

(10) **Patent No.:** **US 6,177,580 B1**

(45) **Date of Patent:** **Jan. 23, 2001**

(54) **CONJUGATED LINOLENIC ACID-BASED  
 SYNTHETIC TRIGLYCERIDES**

(75) **Inventors:** **Franz Timmermann**, Illertissen; **Rolf Gaupp**, Dietenheim; **Juergen Gierke**; **Rainer Von Kries**, both of Illertissen; **Wolfgang Adams**, Meckenbeuren; **Andreas Sander**, Illertissen, all of (DE)

(73) **Assignee:** **Henkel Kommanditgesellschaft auf Aktien**, Duesseldorf (DE)

(\*) **Notice:** Under 35 U.S.C. 154(b), the term of this patent shall be extended for 0 days.

(21) **Appl. No.:** **09/423,054**

(22) **PCT Filed:** **Apr. 21, 1998**

(86) **PCT No.:** **PCT/EP98/02332**

§ 371 Date: **Oct. 29, 1999**

§ 102(e) Date: **Oct. 29, 1999**

(87) **PCT Pub. No.:** **WO98/49129**

**PCT Pub. Date:** **Nov. 5, 1998**

(51) **Int. Cl.<sup>7</sup>** ..... **C07C 51/00**; C07C 3/00;  
 C11C 1/00; C11C 3/00; C07B 35/08

(52) **U.S. Cl.** ..... **554/169**; 554/173; 554/126

(58) **Field of Search** ..... 554/169, 173,  
 554/126

(56) **References Cited**

**U.S. PATENT DOCUMENTS**

3,984,444 10/1976 Ritz et al. .... 260/405.6  
 5,466,843 \* 11/1995 Cooper et al. .... 554/149  
 5,585,399 12/1996 Hong et al. .... 514/546

**FOREIGN PATENT DOCUMENTS**

21 55 727 5/1973 (DE) .  
 0 579 901 1/1994 (EP) .

6-276939 10/1994 (JP) .  
 WO94/16690 8/1994 (WO) .  
 WO96/06605 3/1996 (WO) .  
 WO96/34846 11/1996 (WO) .  
 WO97/18320 5/1997 (WO) .

**OTHER PUBLICATIONS**

Chin et al, Dietary Sources of Conjugated Dienoic Isomers of Linoleic Acid, a Newly Recognized Class of Anticarcinogens, Journal of Food Composition and Analysis, Sep. 1992.\*

Nutrition, vol. 19/ NR.6 (1995).

J. Food Compos. Anal.5, 185-197.

Carcinogenesis 8, 1881-1887 (1987).

Cancer Lett. 63, 125-133 (1992).

Artherosclerosis 108, 19-25 (1994).

Agric. Biol. Chem., 47 (10), 2243-2249 1983.

JAACS, vol. 50 pp. 459-461.

JOACS, vol. 73, No. 11 (1996) pp. 1415-1420.

JAACS, vol. 59, No. 3 (1982) pp. 124-129.

\* cited by examiner

*Primary Examiner*—Deborah D. Carr

*Assistant Examiner*—Diedra Faulkner

(74) *Attorney, Agent, or Firm*—John E. Drach; Steven J. Trzaska

(57) **ABSTRACT**

A process for making synthetic triglycerides involving: (a) providing a reaction component selected from the group consisting of glycerol, a triglyceride, and mixtures thereof; (b) providing a fatty acid mixture containing at least 50% by weight, based on the weight of the fatty acid mixture, of conjugated linoleic acid; (c) providing an inert gas atmosphere; (d) combining the reaction component with the fatty acid mixture, in the inert atmosphere, to form a reaction mixture; and (e) heating the reaction mixture to a temperature of from 180 to 240° C., at a heating rate of from 0.5 to 3 K per minute, thus forming the synthetic triglyceride.

**16 Claims, No Drawings**



# CONJUGATED LINOLENIC ACID-BASED SYNTHETIC TRIGLYCERIDES

## BACKGROUND OF THE INVENTION

This invention relates to synthetic triglycerides containing  $C_{6-24}$  fatty acid residues, with the proviso that at least one residue is a conjugated linoleic acid residue, and to a process for the production of the triglycerides. The invention also relates to the use of the triglycerides in foods and pharmaceutical products.

Polyunsaturated  $\omega$ -3 and  $\omega$ -6 fatty acids, such as  $\alpha$ -linolenic acid and linoleic acid, are among the fatty acids essential to mammals and human beings. Besides linoleic acid, other isomeric octadecadienoic acids occur in nature. They are distinguished by conjugated double bonds at carbon atoms 9 and 11, 10 and 12 and 11 and 13. These isomeric octadecadienoic acids are collectively referred to in the scientific literature as conjugated linoleic acids (abbreviation: CLAs) and have recently attracted increasing attention (NUTRITION, Vol. 19, No. 6, 1995).

Conjugated linoleic acids are present as constituents in various foods. Their main source are animal foods although significant quantities of CLA are also present in milk and milk products. In addition, CLAs have been found in various oils and fats, the concentration in vegetable oils being significantly lower than the concentration in animal fats (J. Food Compos. Anal. 5, 185-197 (1992)).

Various working groups have reported on the significance of CLAs to the organism. Recently, Shultz et al. reported on the inhibiting effect on the in-vitro growth of human cancer cells (Carcinogenesis 8, 1881-1887 (1987) and Cancer Lett. 63, 125-133 (1992)).

In in vitro tests, CLAs were tested for their effectiveness against the growth of malignant human melanomas, colon and breast cancer cells. In the culture media, there was a significant reduction in the growth of the cancer cells treated with CLAs by comparison with control cultures. The mechanism by which CLAs exert anticarcinogenic activity is unknown. In addition, CLAs have a strong antioxidative effect so that, for example, the peroxidation of lipids can be inhibited (Atherosclerosis 108, 19-25 (1994)).

Investigations have also been conducted, for example, into the addition of conjugated linoleic acid to foods for the purpose of color stabilization (JP 06/276939 A2).

The use of conjugated linoleic acid in animal feeds and, in this connection, also in human nutrition is known, for example, from WO 96/06605. This application is concerned with reducing body fat content in animal nutrition. In the statement of problem, the specification also mentions the possibility of application to human beings. In particular, it mentions the use of a fatty emulsion containing 0.5 to 2% by weight of conjugated linoleic acid for oral or intravenous administration to human beings.

EP 0 579 901 B relates to the use of conjugated linoleic acid for avoiding loss of weight or for reducing increases in weight or anorexia caused by immunostimulation in human beings or animals.

WO 94/16690 is concerned with improving the efficiency of food utilization in animals by administering an effective quantity of conjugated linoleic acid.

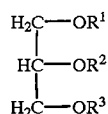
In connection with the many positive effects of conjugated linoleic acid, as demonstrated in intensive studies, particularly on animals and tissue cultures, the use of conjugated linoleic acid in foods for human consumption has also been discussed. However, the use of free conjugated

linoleic acid in foods and pharmaceutical products is limited by the fact that, on the one hand, unwanted reactions with other food constituents can occur where free conjugated linoleic acid is incorporated in complex foods and, on the other hand, by the unpleasant taste and odor of conjugated linoleic acid which can lead to non-acceptance by the consumer. Another disadvantage is that free fatty acids are covered by food additive legislation so that their use in foods is restricted.

Accordingly, the complex problem addressed by the present invention was to find a substitute for conjugated linoleic acid, above all in human nutrition and also for pharmacological use. On the one hand, this substitute would have better organoleptic properties than conjugated linoleic acid and, on the other hand, would lend itself to incorporation in foods without initiating secondary reactions.

## DESCRIPTION OF THE INVENTION

Accordingly, the present invention relates to synthetic triglycerides corresponding to formula (I):



(I)

in which  $R^1$ ,  $R^2$  and  $R^3$  independently of one another represent  $C_{6-24}$  fatty acid residues, with the proviso that at least one substituent  $R^1$ ,  $R^2$  or  $R^3$  is a conjugated linoleic acid residue.

The present invention also relates to a process for the production of synthetic triglycerides by esterification of glycerol or transesterification of triglycerides with fatty acid mixtures by methods known from the prior art, with the proviso that at least 50% by weight of conjugated linoleic acid is used in the fatty acid mixture, the reaction is carried out in an inert gas atmosphere and heating to the reaction temperature is preferably carried out at a heating rate of 0.2 to 10 K per minute.

The present invention also relates to the use of the triglycerides according to the invention in foods and/or as active substances for the production of pharmaceutical products.

It has surprisingly been found that the triglycerides according to the invention are comparable with pure conjugated linoleic acid in their antioxidative and color-stabilizing effect in foods. In addition, they may readily be incorporated in foods and pharmaceutical products without initiating any secondary reactions. In particular, their lipophilic character enables them to be readily incorporated in fat-containing products. The triglycerides according to the invention are also readily absorbed by the animal or human organism. At the same time, they have an almost neutral taste and odor. They are therefore far superior in their organoleptic properties to free conjugated linoleic acid. This means, for example, that the triglycerides can be used in larger quantities in foods. Even food supplements consisting of pure triglyceride of conjugated linoleic acid can be taken by mouth by virtue of their outstanding organoleptic properties. Another advantage is that they do not come under food additive legislation, in other words there are no restrictions on their incorporation in foods.

It has also been found that, surprisingly, the triglycerides according to the invention can also readily be obtained in high yields, more particularly by the direction esterification

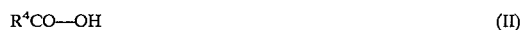
of glycerol with conjugated linoleic acid, providing the reaction is carried out in an inert gas and providing a low heating rate is maintained.

#### Triglycerides

In the triglycerides of formula (I) according to the invention, at least one of the substituents  $R^1$ ,  $R^2$  or  $R^3$  is a conjugated linoleic acid residue while the other constituents represent  $C_{6-24}$  fatty acid residues. However, triglycerides which, on a statistical average, contain more than 2 conjugated linoleic acid residues per triglyceride are particularly preferred, triglycerides in which the substituents  $R^1$ ,  $R^2$  and  $R^3$  represent a conjugated linoleic acid residue being most particularly preferred. In the context of the present invention, triglycerides are also understood to be the technical mixtures of mono-, di- and triglycerides which are obtained in particular in the direct esterification of glycerol with conjugated linoleic acid. A typical composition which is particularly preferred and which is obtained in the process according to the invention, more particularly in the esterification of glycerol with conjugated linoleic acid, contains 60 to 98% by weight and preferably 80 to 98% by weight of a triglyceride of conjugated linoleic acid, 1 to 40 and preferably 1 to 20% by weight of a diglyceride of conjugated linoleic acid and at most 2% by weight and preferably at most 1% by weight of a monoglyceride of conjugated linoleic acid. At the same time, the glyceride to be used in accordance with the invention has an acid value of at most 5 and preferably of at most 3, a hydroxyl value below 40 and preferably below 30 and a peroxide number below 4 and preferably below 2.

#### Fatty Acids

Fatty acids in the context of the present invention are aliphatic carboxylic acids corresponding to formula (II):



in which  $R^4CO$  is an aliphatic, linear or branched acyl group containing 6 to 24 carbon atoms and 0 and/or 1, 2 or 3 double bonds.

Typical examples are caproic acid, caprylic acid, 2-ethylhexanoic acid, capric acid, lauric acid, isotridecanoic acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, isostearic acid, oleic acid, elaidic acid, petroselinic acid, linoleic acid, linolenic acid, elaeostearic acid, arachic acid, gadoleic acid, behenic acid and erucic acid and the technical mixtures thereof obtained, for example, in the pressure hydrolysis of natural fats and oils, in the reduction of aldehydes from Roelen's oxosynthesis or in the dimerization of unsaturated fatty acids.

Technical  $C_{12-18}$  fatty acids such as, for example, coco-fatty acid, palm oil fatty acid, palm kernel oil fatty acid or tallow fatty acid, are preferred.

According to the invention, conjugated linoleic acid is preferably understood to be the main isomers 9,11-octadecadienoic acid and 10,12-octadecadienoic acid, but also includes the isomer mixtures normally obtained in the production of conjugated linoleic acid.

#### Transesterification

The fatty acid glycerides to be used as starting materials in accordance with the invention may be the usual natural vegetable or animal fats and oils. These include, for example, palm oil, palm kernel oil, cottonseed oil, rapeseed oil, coconut oil, peanut oil, olive oil, linseed oil, babassu oil, tea oil, olive kernel oil, meadow foam oil, chaulmoogra oil, coriander oil, soybean oil, castor oil, lard oil, beef tallow, lard, fish oil and also sunflower oil and rapeseed oil from old and new plants. The main constituents of these fats and oils are glycerides of various types of fatty acids which contain

considerable quantities of impurities such as, for example, aldehyde compounds, phospholipid compounds and free fatty acids. These materials may be used either directly or after purification. In some cases, it is particularly advisable to esterify the free fatty acids with lower alcohols in a preliminary reaction. These triglycerides are transesterified with fatty acid mixtures containing at least 50, more especially 70 to 100% by weight of conjugated linoleic acid in an inert gas atmosphere by methods known from the prior art, i.e. by acidic and/or basic transesterification. Nitrogen is preferably used as the inert gas. The reaction is preferably carried out at a temperature of 180 to 240° C. In one particularly preferred embodiment, heating to that temperature is carried out at a low rate of 0.2 to 10 and preferably 0.5 to 3 K per minute. Suitable catalysts are any of the usual, catalysts known from the prior art for esterification or transesterification reactions. Such catalysts are, for example, alkali metal and/or alkaline earth metal alcoholates or hydroxides, more especially sodium methanolate and sodium glycerate. It is also preferred to use acetates, such as zinc and/or magnesium acetate, or even titanates and, in particular, tin compounds, organotin compounds, such as dibutyl tin diacetate for example, or tin salts.

#### Esterification

In one particularly preferred embodiment, the triglycerides according to the invention are not produced by transesterification, but rather by the direct esterification of glycerol with a fatty acid mixture containing at least 50 and preferably 70 to 100% by weight of conjugated linoleic acid in an inert gas atmosphere, again preferably nitrogen, by methods known from the prior art. The esterification of glycerol with 100% by weight of conjugated linoleic acid is particularly preferred. Technical mixtures of mono-, di- and triglycerides of conjugated linoleic acid are normally obtained. According to the invention, these mixtures may be used both directly and after further purification. The foregoing observations on the temperature, heating rate and catalyst used for the transesterification apply similarly to esterification.

After esterification or transesterification, 0.01 to 1% by weight of an antioxidant is preferably added. In another preferred embodiment, the triglyceride obtained is purified using a thin-layer evaporator, after which another 0.01 to 1% by weight of an antioxidant is added.

#### Antioxidants

Antioxidants in the context of the present invention are any of the usual natural antioxidants which are used in particular in pharmaceutical products and in foods, including vitamin C and vitamin C derivatives such as, for example, ascorbyl palmitate, carotinoids, rosemary extracts and/or synthetic antioxidants such as, for example, BHA, BHT, TBHQ or gallates and, in particular, various vitamin E derivatives, such as Coviox® T 70 for example.

#### Uses

The triglycerides according to the invention are particularly suitable for use in foods, preferably so-called functional foods, and in pharmaceutical products, more especially as a supporting agent in the treatment of tumors or even in the treatment of patients suffering from catabolic conditions. Since the physiological properties of the triglycerides according to the invention are comparable with those of free conjugated linoleic acid both in human beings and in animals, the triglycerides are suitable for use in all those areas which are already known from the literature for conjugated linoleic acid.

## EXAMPLES

### Production of a Conjugated Linoleic Acid Triglyceride

#### Example 1

92.1 kg of glycerol and 841.5 kg of conjugated linoleic acid were preheated under nitrogen to around 80° C. and

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introduced into a reactor, after which 0.62 kg of tin grindings were added with stirring. The reactor was then evacuated to 30 mbar, stirred for 10 minutes and blanketed with nitrogen. Heating was carried out under nitrogen at a rate of 1 K per minute, the temperature reaching 150° C. after 1 hour. At the same time, the pressure was reduced to 800 mmbar. The temperature was then increased to 210° C. over a period of another hour and the reaction mixture was stirred at that temperature for 2 hours. The reaction mixture was then re-evacuated to 30 mmbar over a period of 30 minutes and stirred until an acid value of 15 had been reached. The reaction mixture was then cooled in vacuo to 90° C. and purged with nitrogen, after which phosphoric acid was added to precipitate the catalyst. After stirring for 15 minutes and after the addition of Perlite, the reaction mixture was filtered through a filter press into a nitrogen-purged receiver to which 0.1% by weight of Coviox T-70 was added as stabilizer.

#### Example 2

The procedure was as in Example 1, except that the crude product was deodorized in a thin-evaporator at 230° C. in the presence of stripping steam. Another 0.2% by weight of Coviox T-70 was added to the end product for stabilization.

#### Glyceride Composition

#### Example 3

A glyceride prepared as described in Example 1 has the following composition:

triglyceride of conjugated linoleic acid:	95% by weight
diglyceride of conjugated linoleic acid	3% by weight
monoglyceride of conjugated linoleic acid:	2% by weight

The acid value was 2, the hydroxyl value 25 and the peroxide number 2.

What is claimed is:

1. A process for making synthetic triglycerides comprising:

- (a) providing a reaction component selected from the group consisting of glycerol, a triglyceride, and mixtures thereof;

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- (b) providing a fatty acid mixture containing at least 50% by weight, based on the weight of the fatty acid mixture, of conjugated linoleic acid;

- (c) providing an inert gas atmosphere;

- (d) combining the reaction component with the fatty acid mixture, in the inert atmosphere, to form a reaction mixture; and

- (e) heating the reaction mixture to a temperature of from 180 to 240° C., at a heating rate of from 0.5 to 3 K per minute, thus forming the synthetic triglyceride.

2. The process of claim 1 wherein the reaction component is glycerol.

3. The process of claim 1 wherein the reaction component is a triglyceride.

4. The process of claim 1 wherein the fatty acid mixture contains from 70 to 100% by weight, based on the weight of the fatty acid mixture, of conjugated linoleic acid.

5. The process of claim 1 further comprising adding a catalyst to the reaction mixture.

6. The process of claim 1 further comprising adding from 0.01 to 1% by weight, based on the weight of the synthetic triglyceride, of an antioxidant to the synthetic triglyceride.

7. The process of claim 1 further comprising purifying the synthetic triglyceride using a thin-layer evaporator in order to form a purified synthetic triglyceride.

8. The product of the process of claim 1.

9. The product of the process of claim 2.

10. The product of the process of claim 3.

11. The product of the process of claim 4.

12. The product of the process of claim 5.

13. The product of the process of claim 6.

14. The product of the process of claim 7.

15. A food product containing the synthetic glyceride of claim 1.

16. A pharmaceutical product containing the synthetic glyceride of claim 4.

\* \* \* \* \*

# **EXHIBIT E**

(19)



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(54) **Method for production of arachidonic acid**

(57) The present invention relates to a process for producing arachidonic acid. In one embodiment, *Mortierella* sect. *schmuckeri* micro organisms are cultured in fermentation medium, preferably containing a component of a complex nitrogen source. Further disclosed is a food product which includes *Mortierella* sect. *schmuckeri* micro organisms or lipid isolated from such micro organisms to enhance the arachidonic acid content of the food product.

**EP 0 726 321 A2**

## Description

## FIELD OF THE INVENTION

5 The present invention relates to a process for production of arachidonic acid. The present invention also relates to a food product and a method to make the food product containing such arachidonic acid.

## BACKGROUND OF THE INVENTION

10 Arachidonic acid (all-cis-5,8,11,14-eicosatetraenoic acid) is a polyunsaturated fatty acid (PUFA) containing 20 carbon atoms with four double bonds. The double bonds are arranged with the last one located six carbon atoms from the methyl end of the chain. Therefore, arachidonic acid is referred to as an omega-6 fatty acid. Arachidonic acid is one of the most abundant C<sub>20</sub> PUFA's in the human body. It is particularly prevalent in organ, muscle and blood tissues. Arachidonic acid is a direct precursor for a number of circulating eicosenoids, such as prostaglandins, thromboxanes, leukotrienes and prostacyclins, which are important biological regulators. These eicosenoids exhibit regulatory effects on lipoprotein metabolism, blood rheology, vascular tone, leukocyte function, platelet activation and cell growth. The application of arachidonic acid to an infant's diet is particularly important due to the rapid body growth of an infant. Arachidonic acid is an important precursor to many of the eicosanoids which regulate cellular metabolism and growth in infants. It is found naturally in human breast milk but not in most infant formula. In an effort to have infant formula match the long chain fatty acid profile found in breast milk, scientific and food regulatory bodies have recommended that arachidonic acid be added to infant formula, especially in formula utilized for premature infants.

In particular, it is preferable that arachidonic acid containing oil produced for use with infant formula contain little or no other long chain highly unsaturated fatty acids (e.g., eicosapentanoic acid). Such other long chain highly unsaturated fatty acids are not preferred because some of these fatty acids can interfere with the utilization of arachidonic acid by the infant, and/or can inhibit blending of the arachidonic acid-containing oil with other oils to achieve the appropriate ratio of fatty acids matching breast milk or other desired applications. Highly unsaturated fatty acids are defined as fatty acids containing 4 or more double bonds.

Traditional sources of arachidonic acid include poultry eggs, bovine brain tissue, pig adrenal gland, pig liver and sardines. The yield of arachidonic acid, however, is usually less than 0.2% on a dry weight basis. The use of microorganisms capable of producing arachidonic acid *de novo* have been suggested by various investigators, including Kyle, PCT Publication No. WO 92/13086, published August 6, 1992; Shinmen et al., U.S. Patent No. 5,204,250, issued April 20, 1993; Shinmen et al., pp. 11-16, 1989, *Appl. Microbiol. Biotechnol.*, vol. 31; Totani et al., pp. 1060-1062, 1987, *LIPIDS*, vol. 22; Shimizu et al., pp. 509-512, 1992, *LIPIDS*, vol. 27; Shimizu et al., pp. 342-347, 1989, *JAACS*, vol. 66; Shimizu et al., pp. 1455-1459, 1988, *JAACS*, vol. 65; Shimizu et al., pp. 254-258, 1991, *JAACS*, vol. 68; Sajbidor et al., pp. 455-456, 1990, *Biotechnology Letters*, vol. 12; Bajpai et al., pp. 1255-1258, 1991, *Appl. Environ. Microbiol.*, vol. 57; Bajpai, pp. 775-780, 1991, *JAACS*, vol. 68; and Gandhi et al., pp. 1825-1830, 1991, *J. Gen. Microbiol.*, vol. 137. The arachidonic acid productivity by the microorganisms disclosed by prior investigators, however, is less than 0.67 grams per liter per day. Such amounts are significantly less than the amounts of arachidonic acid produced by the microorganisms of the present invention. These lower productivity values are the result of employing strains: (1) with slow growth or lipid production rates leading to long fermentation times (i.e., greater than 2-3 days) ( Kyle, 1992, *ibid.*; Shinmen et al., 1993, *ibid.*; Shinmen et al., 1989, *ibid.*; Bajpai et al., 1991, *ibid.*; Bajpai, *ibid.*; and Gandhi et al., *ibid.*); and/or (2) that contain low arachidonic acid contents (expressed as % fatty acids) in the final oil produced (Shinmen et al., 1993, *ibid.*; Shimizu et al., 1989, *ibid.*; and Kendrick and Ratledge, 1992, pp. 15-20, *Lipids*, vol. 27); and/or (3) which require long periods of stress (i.e., aging a biomass for 6-28 days) to achieve high levels of arachidonic acid in a biomass (Bajpai et al., 1991, *ibid.* and Shinmen et al., 1989, *ibid.*); and/or (4) that only exhibit high arachidonic acid content in non-commercial growth conditions (e.g., malt agar plates) (Totani and Oba, 1987, pp. 1060-1062, *Lipids*, vol. 22). In addition, non-*Mortierella schmuckeri* microorganisms that have been proposed for producing arachidonic acid, in particular *Pythium insidiosum* microorganisms, disclosed by prior investigators (Kyle, 1992, *ibid.*), have been reported to be pathogenic to humans and/or animals.

Thus, there remains a need for an economical, commercially feasible method for producing arachidonic acid. The present invention satisfies that need. There also remains a need for the an economical, commercially feasible food product for the introduction of arachidonic acid produced according to the present invention into the diet of human infants.

## SUMMARY

The present invention provides for a method for economically producing arachidonic acid. One embodiment of the present invention includes a method to produce arachidonic acid, comprising culturing microorganisms of the genus *Mortierella* sect. *schmuckeri* in a medium comprising a source of assimilable organic carbon and a source of assimila-

ble nitrogen. In another embodiment, such strains of *Mortierella* sect. *schnuckeri* are capable of producing at least about 0.86 grams per liter per day of arachidonic acid.

Yet another embodiment of the present invention includes a food product comprising lipids recovered from a microorganism of the genus *Mortierella* sect. *schnuckeri* and a food material. In particular, such lipids can be added to infant formula and baby food to increase the arachidonic acid or long chain omega-6 fatty acid content of such foods.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for a novel process for the production of commercially feasible amounts of arachidonic acid using a *Mortierella* sect. *schnuckeri* microorganism. One embodiment of the present process is to produce arachidonic acid by culturing microorganisms of the genus *Mortierella* sect. *schnuckeri* in a medium comprising a source of assimilable organic carbon and a source of assimilable nitrogen. The lower fungi Phycmycetes contains at least two classes, including Oomycetes and Zygomycetes. The class Zygomycetes contains at least two orders, including Entomophthorales and Mucorales. Contained within the Mucorales order are numerous genera including *Mortierella*. The genus *Mortierella* contains nine sections, including sect. *schnuckeri* (Gams, 1977, pp. 381-391, *Persoonia*, vol. 9 and Gams, 1977, p. 216, in Abstracts Vol. A-L, Second International Mycological Congress, University of South Florida). The *schnuckeri* sect. of the genus *Mortierella* contains three species referred to as *Mortierella camargensis*, *Mortierella clausenii* and *Mortierella schnuckeri*.

All of the other strains of *Mortierella* that have been evaluated for arachidonic acid production belong to the *Mortierella* sections *alpina*, *hygrophila* or *spinosa*. It has now been recognized that strains of *Mortierella* sect. *schnuckeri* are particularly advantageous in the production of arachidonic acid compared to these other strains of *Mortierella*. In particular, it has been found that strains of *Mortierella* sect. *schnuckeri* are capable of producing arachidonic acid with high productivity. Strains of *Mortierella* sect. *schnuckeri* of the present invention are preferably capable of producing at least about 0.70 grams of arachidonic acid per liter per day, more preferably at least about 0.80 grams of arachidonic acid per liter per day, and even more preferably at least about 0.86 grams of arachidonic acid per liter per day. Preferably, strains of *Mortierella* sect. *schnuckeri* of the present invention are also capable of producing a total fatty acid content of at least about 20% of dry weight, preferably at least about 30% of dry weight, and more preferably at least about 40% of dry weight. Moreover, preferred strains of *Mortierella* sect. *schnuckeri* of the present invention contain at least about 20% of total fatty acids as arachidonic acid, more preferably at least about 35% total fatty acids as arachidonic acid, and even more preferably at least about 48% total fatty acids as arachidonic acid. The arachidonic acid content of cellular biomass of strains of *Mortierella* sect. *schnuckeri* of the present invention can be at least about 5% of cellular dry weight, preferably at least about 8% of cellular dry weight, and more preferably at least about 13% of cellular dry weight.

Oil recovered, such as by extraction, from a preferred strain of *Mortierella* sect. *schnuckeri* of the present invention contains at least about 20% arachidonic acid, more preferably at least about 30% arachidonic acid, and even more preferably at least about 41% arachidonic acid. As used herein, "lipid" "lipid extract", "oil" and "oil extract" are used interchangeably.

Morphological growth forms of fungi can have a significant effect on growth and product formation in fermenters. Fungal morphology in fermenters can range from a dispersed filamentous form to a dense pellet form. Species of *Mortierella* sect. *schnuckeri* of the present invention have an advantage over previously utilized species of *Mortierella*, including the ability to readily grow (early in a fermentation) in a dispersed filamentous form when grown in agitated liquid cultures such as shake flasks or fermenters. Some other species of *Mortierella* grown in fermentation medium typically grow in the form of pellets or spherical aggregates (i.e., having the appearance of a very tight cotton ball), sometimes exhibiting a dispersed form only after several days in a fermentation. Without being bound by theory, it is believed that the growth and productivity of cells in the pellet form is limited because cells in the center of a pellet or aggregate are not exposed to the necessary nutrients contained in the fermentation medium. Traditional methods of growing these fungal populations can include increasing the agitation of the fermenter or addition of detergents in an attempt to disperse such aggregates and improve cell growth. The present inventor has discovered that strains of *Mortierella* sect. *schnuckeri* of the present invention readily grow in the dispersed filamentous form, thereby improving growth and productivity of such cells by enabling nutrients to reach all the cells. As used herein, the term "filamentous" refers to the growth of fungi as a loosely branched network of short mycelia rather than as a pellet or aggregate.

Preferred strains of *Mortierella* sect. *schnuckeri* of the present invention include strains of *Mortierella* sect. *schnuckeri* isolated from cold, arid soil, in which the microorganisms experience short periods of wetness. In particular, such areas can include soils that experience some prolonged periods of freezing or near freezing conditions. More preferred strains of *Mortierella* sect. *schnuckeri* are isolated from the Southwest region of North America, in particular, desert regions of the United States and/or Mexico. In particular, strains of *Mortierella* sect. *schnuckeri* of the species *Mortierella schnuckeri* are isolated from southern California and/or Mexico.

Strains of *Mortierella* can be isolated from soils or aquatic habitats using techniques known in the art (Stevens, 1974, in *Mycology Guidebook*, University of Washington Press, Seattle; and Barron, pp. 405-427, 1971, in *Methods of*

*Microbiology*, Vol. 4.). More specifically, species of *Mortierella* sect. *schnuckeri* can be isolated by suspending small samples of soil in distilled water and then streaking a portion of the suspension on corn meal agar plates or agar plates containing a desired fermentation media. Additionally, species of *Mortierella* sect. *schnuckeri* can be isolated from aquatic habitats using techniques known in the art (see, for example, U.S. Patent No. 5,130,242, by Barclay et al., issued July 14, 1992; and U.S. Patent No. 5,340,594, by Barclay et al., issued August 23, 1994). On agar plates, *Mortierella* colonies can be partly identified by several characteristics, including for example, as white colored colonies which grow essentially within the agar rather than predominantly exhibiting aerial growth. *Mortierella* colonies can also be distinguished from other fungi using the general characteristics of fungal taxonomy outlined, for example, by Talbot (Principles of Fungal Taxonomy, 1971, Macmillan Press). After isolation of a pure colony, members of the genus *Mortierella* can also be identified by, for example, a garlic-like odor when cultured in a shake flask or in agar plate cultures containing media described in Stevens, *ibid*. The culture producing the best sporulation can then be used to identify the species of the culture using the *Mortierella* keys outlined in Gams (pp. 381-391, 1977, *Persoonia*, Vol. 9; and in *Taxonomic problems in Mortierella*, Abstracts, 2nd International Mycological Conference, University of South Florida, Tampa, published by Hamilton Newell, Inc., Amherst, MA).

After isolation of a pure colony of strains of *Mortierella* sect. *schnuckeri*, the biomass of the strain can be analyzed for lipid content and arachidonic acid content by gas chromatography. Preferred colonies that exhibit rapid growth and high lipid and high arachidonic acid content can then be selected. Further selection for the presence or absence of other characteristics can also be conducted. For example, in the application of extracted lipids in infant formula for the benefit of arachidonic acid content, the presence of eicosapentanoic acid (C20:5n-3; "EPA") is detrimental. Therefore, one can select for the absence of high EPA content. One preferred species of *Mortierella* sect. *schnuckeri* of the present invention is *Mortierella camargensis*. Particularly preferred strains of *Mortierella camargensis* of the present invention have the identifying characteristic of being able to produce about 0.86 grams of arachidonic acid per liter per day. Another identifying characteristic is that between about 25% and about 33% of the total fatty acids produced by such particularly preferred *Mortierella camargensis* can be arachidonic acid. Thus, the resulting arachidonic acid content of a biomass of a particularly preferred *Mortierella camargensis* of the present invention can be between about 9.6% and about 10.8% under appropriate fermentation conditions. Yet another identifying characteristic is that the resulting oil recovered from a particularly preferred *Mortierella camargensis* of the present invention can have an arachidonic acid content ranging from about 20% to about 30% of the total fatty acids.

Another particularly preferred species of *Mortierella* sect. *schnuckeri* of the present invention, *Mortierella schnuckeri* has the identifying characteristic of being able to produce about 0.84 grams of arachidonic acid per liter per day. Another identifying characteristic is that between about 40% and about 49% of the total fatty acids produced by such particularly preferred *Mortierella schnuckeri* of the present invention can be arachidonic acid. Thus, the resulting arachidonic acid content of a biomass of a particularly preferred *Mortierella schnuckeri* of the present invention can be between about 12.5% and about 13.6% under appropriate fermentation conditions. Yet another identifying characteristic is that the resulting oil recovered from a particularly preferred *Mortierella schnuckeri* can have an arachidonic acid content ranging from about 33% to about 41% of the total fatty acids.

It is within the scope of the present invention that, in addition to known strains of *Mortierella* sect. *schnuckeri*, such as those on deposit with the American Type Culture Collection (e.g., ATCC), newly identified strains from nature and mutant strains derived from known or newly identified strains, can be used to produce arachidonic acid. Naturally-occurring mutants of a parental strain of *Mortierella* sect. *schnuckeri* that are capable of producing arachidonic acid can be isolated by, for example, subjecting a parental strain to at least one round of chemical or physical mutagenesis in order to increase the rate of mutagenesis, thereby increasing the probability of obtaining a microorganism producing increased amounts of arachidonic acid. It will be obvious to one skilled in the art that mutant microorganisms of the present invention also include arachidonic acid-producing microorganisms that can be obtained by genetically-engineering microorganisms to produce increased amounts of arachidonic acid. For example, it is within the purview of the present invention to transform *Mortierella* sect. *schnuckeri* microorganisms with nucleic acid molecules encoding enzymes of the arachidonic acid biosynthetic pathway obtained from fungal arachidonic acid-producing microorganisms, such as those of the genus *Mortierella* sect. *schnuckeri*. A *Mortierella* sect. *schnuckeri* nucleic acid molecule of the present invention can be obtained from its natural source either as an entire (i.e., complete) gene or a portion thereof capable of forming a stable hybrid with the entire gene. A nucleic acid molecule from a strain of *Mortierella* sect. *schnuckeri* can also be produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. As used herein, a "mutated microorganism" is a mutated parental microorganism in which the nucleotide composition of such microorganism has been modified by mutation(s) that occur naturally, that are the result of exposure to a mutagen, or that are the result of genetic engineering.

Preferred mutants of strains of *Mortierella* sect. *schnuckeri* of the present invention have one or more of the identifying characteristics of a preferred *Mortierella camargensis* of the present invention and a preferred *Mortierella schnuckeri* of the present invention as described in detail above.

In accordance with the present invention, microorganisms of the genus *Mortierella* sect. *schnuckeri* capable of producing arachidonic acid, are cultured in an effective medium, herein defined as any medium capable of promoting ara-



chidonic acid production. Preferably, the effective medium also promotes rapid fungal growth. The microorganisms of the genus *Mortierella* sect. *schmuckeri* of the present invention can be cultured in conventional fermentation modes, which include, but are not limited to, batch, fed-batch, and continuous.

5 The present invention provides a method to produce arachidonic acid, comprising culturing microorganisms of the genus *Mortierella* sect. *schmuckeri* in a medium comprising a source of assimilable organic carbon and a source of assimilable nitrogen.

Sources of assimilable carbon include but are not limited to sugars and their polymers, including starches, dextrin, saccharose, maltose, lactose, glucose, fructose, mannose, sorbose, arabinose, xylose, levulose, cellobiose, and molasses; fatty acids; and polyalcohols such as glycerine. Preferred carbon sources in the present invention include  
10 monosaccharides, disaccharides, and trisaccharides. The most preferred carbon source is glucose.

Sources of assimilable nitrogen useful for fermentation of a microorganism of the present invention include simple nitrogen sources, organic nitrogen sources and complex nitrogen sources. Such nitrogen sources include ammonium salts and substances of animal, vegetable and/or microbial origin. Such organic nitrogen sources include corn steep liquor, protein hydrolysates, microbial biomass hydrolysates, soy tone, soy meal, fish meal, meat meal, meat extract, peptone, tryptone, yeast extract, yeast, whey, ammonium sulfate, urea, ammonium nitrate and amino acids.  
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Preferred nitrogen sources for use in an effective medium of the present invention include complex nitrogen sources. Use of a complex nitrogen source in a fermentation medium of the present invention increases arachidonic acid production by a strain of *Mortierella* sect. *schmuckeri* of the present invention by at least about 50 percent and preferably by at least about 100 percent, either as measured by percent dry weight or percent total fatty acids in an oil, compared with a strain of *Mortierella* sect. *schmuckeri* grown in the absence of a complex nitrogen source. Suitable complex nitrogen sources include, for example, corn steep liquor, protein hydrolysates, microbial biomass hydrolysates, soy tone, soy meal, fish meal, meat meal, meat extract, peptone, tryptone, yeast extract, yeast and whey. One of skill in the art can determine which complex nitrogen source best stimulates arachidonic acid production in the strain of *Mortierella* sect. *schmuckeri* employed in a fermentation process.  
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In a preferred embodiment of the present invention, a fermentation is conducted in which a non-carbon nutrient, for example, nitrogen or magnesium and preferably nitrogen, is limited. In this manner, cellular metabolism is directed towards lipid production, thus enhancing the overall production of arachidonic acid.  
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The effective medium can contain other compounds such as inorganic salts, vitamins, trace metals or growth promoters. Such compounds can be present in carbon, nitrogen, or mineral sources in the effective medium or can be added specifically to the medium. Low concentrations of magnesium are also preferred.  
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During the fermentation, variables including the oxygen content, pH, temperature, carbon dioxide content, and rate of carbon source addition are controlled to maximize the production of arachidonic acid without unduly limiting the length of time during which successful fermentation can be accomplished. The optimum oxygen concentration for arachidonic acid production can be determined for any particular population of *Mortierella* sect. *schmuckeri* by variation of the oxygen content of the medium. In particular, the oxygen content of the fermentation medium is maintained at an oxygen content preferably ranges from between about 20% of saturation and about 60% of saturation.  
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Growth of strains of *Mortierella* sect. *schmuckeri* of the present invention can be effected at any temperature conducive to satisfactory growth of the strain; for example, between about 25°C and about 33°C, preferably between about 27°C and about 32°C, and more preferably at about 30°C. The culture medium typically becomes more alkaline during the fermentation if pH is not controlled by acid addition or buffers. The strains of *Mortierella* sect. *schmuckeri* of the present invention will grow over a pH range from between about 4.0 to about 10.0 with a starting pH of about 5.5 being more preferred.  
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Another aspect of the present invention includes a food product comprising a food material combined with microorganisms of the genus *Mortierella* sect. *schmuckeri*. Strains of *Mortierella* sect. *schmuckeri* of the present invention are added to a food material to create a food product having enhanced concentrations of arachidonic acid. As used herein, the term "food material" refers to any food type fed to humans or non-human animals. Also within the scope of the present invention is a method to make a food product comprising adding microorganisms of the genus *Mortierella* sect. *schmuckeri* to a food material.  
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*Mortierella* sect. *schmuckeri* of the present invention are recovered for use as a food supplement simply by separating the cells from fermentation medium. A variety of procedures can be employed in the recovery of microbial cells from the culture medium. In a preferred recovery process, the cells produced in the fermentation process are recovered from the culture medium by separation using conventional means, such as centrifugation or filtration. The cells can then be washed, frozen, lyophilized, and/or dried (e.g., spray drying, tunnel drying, vacuum drying, or a similar process). The arachidonic acid rich oil can be extracted immediately from the cells or the resulting cells can then be stored under a non-oxidizing atmosphere of a gas such as N<sub>2</sub> or CO<sub>2</sub> (to eliminate the presence of O<sub>2</sub>) prior to incorporation into a food material. Alternatively, recovered cells can be used directly (without drying) as a feed supplement. To extend its shelf life, the wet biomass of a strain of *Mortierella* sect. *schmuckeri* can be acidified (approximate pH = 3.5-4.5) and/or pasteurized or flash heated to inactivate enzymes and then canned, bottled or packaged under a vacuum.  
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A suitable food material useful for the formation of a food product of the present invention includes animal food. The term "animal" means any organism belonging to the kingdom Animalia and includes, without limitation, primates (e.g., humans and monkeys), livestock and domestic pets. The term "food product" includes any product to be fed to such animals. Preferred food materials to be consumed by humans includes infant formula and baby food. Preferred food materials to be consumed by domestic pets includes dog foods. By adding *Mortierella* sect. *schmuckeri* biomass or extracted oil to provide a source of arachidonic acid, preferred food products of the present invention comprise a total fatty acid content in which up to about 20% by weight of total fatty acids is arachidonic acid, more preferred food products of the present invention comprise a total fatty acid content in which up to about 10% by weight of total fatty acids is arachidonic acid, and even more preferred food products of the present invention comprise a total fatty acid content in which between about 0.1% and about 1.0% by weight of total fatty acids is arachidonic acid.

A further embodiment includes a food product comprising lipids recovered from a microorganism of the genus *Mortierella* sect. *schmuckeri* and a food material. Recovered lipids can include either all of the lipids recovered from the microorganisms or a portion thereof (i.e., isolated arachidonic acid or total fatty acids containing arachidonic acid). In the former instance, the lipid composition includes arachidonic acid in about the same relative amount as it exists in the organism. Alternatively, the recovered lipids can be further processed to concentrate the arachidonic acid to achieve a composition having a greater concentration of arachidonic acid than occurs naturally in the organism. Also within the scope of the present invention is a method to make a food product comprising adding lipids recovered from a microorganism of the genus *Mortierella* sect. *schmuckeri* to a food material.

Recovery of lipids from strains of *Mortierella* sect. *schmuckeri* can be accomplished by any suitable method, including numerous methods known in the art. For example, recovery can include the following method. Harvested cells (fresh or dried) can be ruptured using techniques known to those in the art. Lipids can then be extracted from the cells by any suitable means, such as by supercritical fluid extraction, or by extraction with solvents such as chloroform, hexane, methylene chloride, methanol, isopropyl, ethyl acetate, and the like, and the extract evaporated under reduced pressure to produce a sample of concentrated lipid material. Arachidonic acid can be further separated from other lipids by chilling a fatty acid composition such that the saturated fatty acids in the composition precipitate out while the arachidonic acid remains in solution. The solution can then be recovered.

The *Mortierella* sect. *schmuckeri* microorganisms can also be broken or lysed and the lipids recovered into edible oil using standard methods known in the art. The recovered oils can be refined by well-known processes routinely employed to refine vegetable oils (e.g., chemical or physical refining). These refining processes remove impurities from recovered oils before they are used or sold as edible oils. The refining process consists of a series of processes to degum, bleach, filter, deodorize and polish the recovered oils. After refining, the oils can be used directly as a feed or food additive to produce arachidonic acid enriched products. Alternatively, the oil can be further processed and purified as outlined below and then used in the applications as described herein.

Lipids recovered from the biomass of a strain of *Mortierella* sect. *schmuckeri* of the present invention can be combined with any animal food material, particularly food materials for humans, to create a food product having enhanced concentrations of arachidonic acid. The amount of fatty acids naturally in food products varies from one food product to another. A food product of the present invention can have a normal amount of arachidonic acid or a modified amount of arachidonic acid. In the former instance, a portion of the naturally occurring lipids are substituted by lipids of the present invention. In the latter instance, naturally occurring lipids are supplemented by lipids of the present invention.

Preferably, lipids recovered from strain of *Mortierella* sect. *schmuckeri* are added to foods for infants, such as infant formula and baby food. According to the present invention, an infant refers to infants in utero and children less than about two years old, including, in particular, premature infants. Arachidonic acid is a particularly important component of infant formula and baby food because of the rapid growth of infants (i.e., doubling or tripling in weight during the first year of life). An effective amount of arachidonic acid to supplement infant formula is an amount that approximates the concentration of arachidonic acid in human breast milk. Preferred amounts of arachidonic acid to add to infant formula or baby food range from between about 0.1 to about 1.0% of total fatty acids, more preferably from between about 0.1 to about 0.6% of total fatty acids, and even more preferably about 0.4% of total fatty acids.

Arachidonic acid produced by the method of the present invention is suitable for use as therapeutic and experimental agents. An embodiment of the present invention comprises the production of arachidonic acid for treatment of arachidonic acid-deficient infants. The arachidonic acid can be included in a parenteral formulation that can be administered to an infant through parenteral routes to fortify the infant's supply of arachidonic acid. Preferred parenteral routes include, but are not limited to, subcutaneous, intradermal, intravenous, intramuscular and intraperitoneal routes. A parenteral formulation can include arachidonic acid of the present invention and a carrier suitable for parenteral delivery. As used herein, a "carrier" refers to any substance suitable as a vehicle for delivering a molecule or composition to a suitable *in vivo* site of action. Examples of such carriers include, but are not limited to water, phosphate buffered saline, Ringer's solution, dextrose solution, serum-containing solutions, Hank's solution and other aqueous physiologically balanced solutions. Acceptable protocols to administer arachidonic acid in an effective manner include individual dose size, number of doses, frequency of dose administration, and mode of administration. Determination of such protocols can be accomplished by those skilled in the art depending upon a variety of variables, including the weight of the

infant and the extent of arachidonic acid deficiency. Another embodiment of the present invention comprises the production of arachidonic acid for treatment of adults, in particular pregnant mothers. Acceptable protocols for administration of arachidonic acid to adults includes parenteral feeding techniques or encapsulating oil recovered from a microorganism of the present invention in a capsule, such as gelatin (i.e., digestible) capsule, for oral administration and/or in a liquid diet formulation. A liquid diet formulation can comprise a liquid composition containing nutrients suitable for supplementing a diet or nutrients sufficient as a complete diet.

Another embodiment of the present invention comprises the production of arachidonic acid for use as an experimental reagent to identify regulators of metabolic pathways for which arachidonic acid is a precursor. For example, arachidonic acid is a precursor for leukotrienes. Leukotrienes are believed to be involved in the occurrence of certain diseases involving inflammation and allergy. As such, inhibitors of leukotriene production may be valuable therapeutic agents. Arachidonic acid recovered using the method of the present invention can be used to test putative inhibitory agents *in vitro* by incubating the putative inhibitor with arachidonic acid under suitable conditions well-known to those of skill in the art, and measuring leukotriene production.

The following examples and test results are provided for the purposes of illustration and are not intended to limit the scope of the invention.

## EXAMPLES

### Example 1

This example describes the production of arachidonic acid by the strain S12 of *Mortierella* sect. *schmuckeri* which is a strain of *Mortierella schmuckeri*.

A strain of *Mortierella schmuckeri* was identified in accordance with the method of the present invention. Such strain is referred to herein as strain S12. A one centimeter squared portion of a *Mortierella schmuckeri* strain S12 was cut from a solid agar plate and placed in 100 ml aliquots of medium containing 10 grams/liter (g/l) of corn steep liquor, 0.1 g/l  $\text{CaCO}_3$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g/l  $\text{KH}_2\text{PO}_4$ , 1 milliliter per liter (ml/l) PII metals (6.0 g  $\text{Na}_2\text{EDTA}$ ; 0.24 g  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ; 6.84 g  $\text{H}_3\text{BO}_3$ ; 0.86 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ; 0.133 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.026 g  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ; 0.005 g  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ ; 0.002 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 0.052 g  $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ ; dissolved in 1 liter of water and pH adjusted to 8.0), and 1 ml/l vitamin mix (100 mg/L thiamin; 500  $\mu\text{g/L}$  biotin and 500  $\mu\text{g/L}$  vitamin  $\text{B}_{12}$ ), contained in 250 ml baffled shake flasks. The cultures were incubated for 72 hours, at 30°C on a rotary shaker (225 rpm). After 72 hours, the cultures were of high density and had stopped growing.

The cells in the flasks were then sampled to determine ash-free dry weights and to quantify the fatty acid content of the cells. The cells of *Mortierella schmuckeri* strain S12 were harvested and centrifuged. Fatty acids in dry biomass of harvested cells were then methylated in 4% methanolic  $\text{H}_2\text{SO}_4$  (4 ml  $\text{H}_2\text{SO}_4$  in 96 ml methanol) at 100°C for 1 hour. The fatty acid methyl esters were then quantified by gas chromatography (Varian 3500 gas chromatograph, Supelco SP 2330 column; initial column temp. = 70°C; detector temp = 250°C; injector temp = 220°C; carrier gas = helium; temperature program: initial column temp = 70°C for 3 min, 20°C per min to 195°C, then hold for 5 min., then 25°C per min to 220°C, then hold for 8 min.). The composition of the fatty acids in the fungal biomass is shown in Table 1.

TABLE 1

S12 Fatty Acid Profile			
FATTY ACID		Fatty Acid Content	
		mg/g dwt*	% TFA*
MYRISTATE	C14:0	0.9	0.3
MYRISTOLEATE	C14:1	1.1	0.3
PALMITATE	C16:0	45.6	13.5
PALMITOLEATE	C16:1	2.2	0.6
STEARATE	C18:0	26.9	8.0
OLEATE	C18:1	39.8	11.8
LINOLEATE	C18:2N6	36.5	10.8
Gamma-LINOLENATE	C18:3N6	13.8	4.1
LINOLENATE	C18:3N3	1.6	0.5
EICOSENOATE-11	C20:1	1.1	0.3
EICOSADIENOATE-11,14	C20:2	2.2	0.6
HOMOGAMMA LINOLENATE	C20:3N6	1.6	0.5
BEHENATE	C22:0	17.4	5.2
ARACHIDONATE	C20:4	135.7	40.3
LIGNOCERATE	C24:0	9.6	2.8
NERVONATE	C24:1	0.6	0.2
		336.5	100.0

\* TFA = Total Fatty Acids

\* dwt = cellular dry weight

The results indicated that under these fermentation conditions, the strain S12 biomass contained about 33.7% of fatty acids. Approximately 40.3% of the total fatty acids was comprised of arachidonic acid. The arachidonic acid content of the biomass therefore was 13.6% cellular dry weight.

### Example 2

This example describes the effect of varying the carbon to nitrogen ratio in the fermentation medium on arachidonic acid production in the fermentation of *Mortierella schmuckeri* strain S12 cells.

Fermentation cultures were prepared as described in Example 1. Numerous fermentation samples were prepared that had increasing concentrations of glucose (the amounts are shown in Table 2, first column). The relative amounts of total fatty acids and arachidonic acid were measured according to the method described in Example 1 and the results are illustrated in Table 2.

TABLE 2

Strain S12: Effect of C:N Ratio on Dry Weight, Lipid and Arachidonic Acid Yields						
glucose g/L	C:N Ratio	Biomass dry wt. g/L	Final pH	Tot. FA % dry wt.	Arachidonic % Tot. FA.	Arachidonic % dry wt.
3.7	3:1	3.1	7.3	15.9	48.6	7.7
6.2	5:1	4.0	7.1	24.0	43.4	10.4
12.4	10:1	6.0	6.6	31.3	35.5	11.1
37.2	30:1	6.2	6.5	31.5	35.6	11.2
49.6	40:1	6.6	6.5	32.0	37.5	12.0
74.4	60:1	6.8	6.4	30.9	39.3	12.1
99.2	80:1	5.8	6.4	25.7	37.8	9.7
124.0	100:1	5.9	6.4	25.5	37.4	9.5

The results indicated that optimal carbon to nitrogen ratio for the fermentation of strain S12 is about 40:1 to about 60:1. The results also indicate that the amount of arachidonic acid produced by S12 cells can be increased by limiting the amount of non-carbon nutrients, in particular nitrogen, in the fermentation medium.

### Example 3

This example illustrates the effect of nutrient manipulation on arachidonic acid production by *Mortierella schmuckeri* strain S12 and *Mortierella camargensis* strain S3.

A strain of *Mortierella camargensis* was identified in accordance with the method of the present invention. Such strain is referred to herein as strain S3. Fermentation cultures were prepared as described in Example 1. Numerous fermentation samples were prepared that had different nutrients deleted from the fermentation medium. The nutrients deleted from the various fermentation samples are shown in Table 3 (first column). The relative amounts of total fatty acids and arachidonic acid were measured according to the method described in Example 1. The results are shown in Table 3.

TABLE 3

Strains S12 and S3: Evaluation of nutrient subtraction on ARA production					
Strain S12: <i>M. schmuckeri</i>					
Nutrient Deleted	Biomass dwt yield g/L	Fatty acid % dwt	ARA % TFA	ARA % dwt	ARA g/L
CaCO <sub>3</sub>	4.2	31.3	31.0	9.7	0.41
Vitamins	5.6	32.8	34.4	11.3	0.63
MgSO <sub>4</sub>	5.4	32.1	39.3	12.6	0.68
PII	5.5	30.6	34.3	10.5	0.58
KH <sub>2</sub> PO <sub>4</sub>	5.5	30.3	35.3	10.7	0.59
Strain S3: <i>M. camargensis</i>					
Nutrient Deleted	Biomass dwt yield g/L	Fatty acid % dwt	ARA % TFA	ARA % dwt	ARA g/L
CaCO <sub>3</sub>	4.5	37.4	21.9	8.2	0.37
Vitamins	5.5	34.9	24.9	8.7	0.48
MgSO <sub>4</sub>	5.4	38.7	25.3	9.8	0.53
PII	5.5	34.9	23.2	8.1	0.45
KH <sub>2</sub> PO <sub>4</sub>	5.3	34.1	23.2	7.9	0.42

The results indicated that, for both strains of *Mortierella* sect. *schmuckeri*, minimizing the magnesium concentration in the fermentation medium had a greater effect on arachidonic acid production than deletion of calcium, vitamins, trace metals and potassium phosphate. For example, the amount of arachidonic acid produced by cells of strain S12 grown in the absence of magnesium was about 0.7 grams of arachidonic acid per liter, while the arachidonic acid production by cells of strain S12 grown in the absence of calcium was on average about 0.4 grams of arachidonic acid per liter.

#### Example 4

This example describes a comparison of arachidonic acid production by *Mortierella camargensis* strain S3 between cells grown in the presence or absence of corn steep liquor, a complex nitrogen source.

A first fermentation sample was prepared using the method and culture medium described in Example 1. A second fermentation sample was prepared using the medium described in Example 1 but instead of corn steep liquor, yeast extract was used as the nitrogen source. Lipids were prepared from strain S3 cells and analyzed using the method described in Example 1. The composition of the fatty acid mixture obtained from each of the foregoing fermentation procedures is shown in Tables 4 and 5. The S3 sample grown with corn steep liquor was found to contain 35.9% of dry weight as fatty acids. The arachidonic acid content of this sample was 10.8% of cellular dry weight. The S3 sample grown without corn steep liquor was found to contain 19.8% of dry weight as of fatty acids. The arachidonic acid content of the sample was 4.8% of cellular dry weight.

TABLE 4

S3 Strain Grown With Corn Steep Liquor			
FATTY ACID		Fatty Acid Content	
		mg/g dwt*	% TFA*
MYRISTATE	C14:0	1.8	0.5
MYRISTOLEATE	C14:1	0.9	0.3
PALMITATE	C16:0	60.1	16.7
PALMITOLEATE	C16:1	1.0	0.3
STEARATE	C18:0	30.3	8.4
OLEATE	C18:1	27.9	7.8
LINOLEATE	C18:2N6	51.4	14.3
GAMMA-LINOLENATE	C18:3N6	27.5	7.7
EICOSENOATE-11	C20:1	1.5	0.4
EICOSADIENOATE-11,14	C20:2	3.1	0.9
HOMOGAMMA LINOLENATE	C20:3N6	1.8	0.5
BEHENATE	C22:0	28.2	7.8
EICOSATRIENOATE	C20:3	0.6	0.2
ARACHIDONATE	C20:4	107.8	30.0
EICOSAPENTANOATE	C20:5N3	0.4	0.1
LIGNOCERATE	C24:0	13.6	3.8
NERVONATE	C24:1	0.8	0.2
DOCOSAHEXANOATE	C22:6N3	0.6	0.2
		359.4	100.0

\* TFA = Total Fatty Acids

\* dwt = cellular dry weight

TABLE 5

S3 Strain Grown Without Corn Steep Liquor			
FATTY ACID		Fatty Acid Content	
		mg/g dwt*	% TFA*
MYRISTATE	C14:0	1.0	0.5
MYRISTOLEATE	C14:1	1.2	0.6
PALMITATE	C16:0	38.9	19.7
PALMITOLEATE	C16:1	0.8	0.4
STEARATE	C18:0	9.7	4.9
OLEATE	C18:1	33.6	17.0
LINOLEATE	C18:2N6	28.1	14.2
GAMMA-LINOLENATE	C18:3N6	11.8	6.0
EICOSENOATE-11	C20:1	1.9	0.9
EICOSADIENOATE-11,14	C20:2	1.0	0.5
HOMOGAMMA LINOLENATE	C20:3N6	4.4	2.2
BEHENATE	C22:0	7.0	3.6
ARACHIDONATE	C20:4	48.8	24.7
ERUCATE	C22:1	0.0	0.0
EICOSAPENTANOATE	C20:5N3	0.0	0.0
LIGNOCERATE	C24:0	8.7	4.4
NERVONATE	C24:1	0.4	0.2
DOCOSAHEXANOATE	C22:6N3	0.4	0.2
		197.7	100.0

\* TFA = Total Fatty Acids

\* dwt = cellular dry weight

The results indicated that inclusion of corn steep liquor as a nitrogen source in the fermentation medium enhanced arachidonic acid production by S3 cells about two-fold. For example, S3 cells grown in the presence of corn steep liquor produced about 107.8 milligrams of arachidonic acid per gram of fungal biomass. Arachidonic acid comprised about 30% of the total fatty acids. Conversely, S3 cells grown in the absence of corn steep liquor produced about 48.8 milligrams of arachidonic acid per gram of fungal biomass. Arachidonic acid comprised about 24.7% of the total fatty acids. Thus, fermentation in the presence of corn steep liquor (a complex nitrogen source) enhanced the production of arachidonic acid.

The results indicate that corn steep liquor is one of the best complex nitrogen sources for stimulating arachidonic acid production in Strain S3. Arachidonic acid production, however, by Strain S12 (*Mortierella schmuckeri*) is stimulated by a wider range of complex nitrogen including, but not limited to, corn steep liquor, yeast extract, yeast, whey and soy flour.

#### Example 5

This example describes a comparison between the arachidonic acid content of *Mortierella camargensis* strain S3 and *Mortierella schmuckeri* strain S12 with previously known ATCC strains of the *schmuckeri* section of *Mortierella*.

Four strains, *Mortierella camargensis* strain S3, *Mortierella schmuckeri* strain S12 and *Mortierella schmuckeri* (ATCC No. 42658) were cultured in the presence or absence of corn steep liquor as described in Example 4. The fatty



acid content of the cells of the S3 strain and the two known strains was measured according to the method described in Example 1. A comparison of the total fatty acid yield and arachidonic acid yields is shown below in Table 6.

TABLE 6

Comparison of Arachidonic Acid and Total Fatty Acid Production in Strains of <i>Mortierella</i> from the Schmuckeri Group of this Fungus		
Total Fatty Acids (as % dwt)	w/o csl	w/ csl
<i>Mortierella camargensis</i> (Strain S3)	19.8	35.9
<i>Mortierella schmuckeri</i> (Strain S12)	18.3	33.7
<i>Mortierella schmuckeri</i> (ATCC 42658)	28.2	38.0
Arachidonic Acid (as % dwt)	w/o csl	w/ csl
<i>Mortierella camargensis</i> (Strain S3)	4.9	10.7
<i>Mortierella schmuckeri</i> (Strain S12)	6.0	13.6
<i>Mortierella schmuckeri</i> (ATCC 42658)	2.5	2.4
Arachidonic Acid (as % total fatty acids)	w/o csl	w/ csl
<i>Mortierella camargensis</i> (Strain S3)	24.7	30.0
<i>Mortierella schmuckeri</i> (Strain S12)	32.6	40.3
<i>Mortierella schmuckeri</i> (ATCC 42658)	9.0	6.3
w/o csl = without corn steep liquor w/ csl = with corn steep liquor % dwt = percent dry weight of biomass		

From the results shown in Table 6 it can be seen that the presence of corn steep liquor in the fermentation medium increases the total fatty acid production by about 2-fold in strain S3 and S12, and by about one third in *Mortierella schmuckeri* (ATCC No. 42658). However, while the presence of corn steep liquor in the fermentation medium increased arachidonic acid content (as %dwt) by about 2-fold in strain S3, the corn steep liquor did not effect arachidonic acid production by *Mortierella schmuckeri* (ATCC No. 42658). *Mortierella clausenii* (ATCC No. 64864) showed no significant growth in either the presence or absence of corn steep liquor.

#### Example 6

This example describes the analysis of the fermentation productivity and lipid content of oil obtained from the *Mortierella schmuckeri* strain S12.

Fermentations were conducted using *Mortierella schmuckeri* strain S12 in two 14 liter fermentation cultures designated vessel B20 and B23. M-3 medium was utilized in vessel B20 and M-6 medium was utilized in vessel B23. M-3 medium contained 12 g/L Cargill 200/20 soy flour, 0.1 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g/L  $\text{CaCO}_3$ , 1 ml/L of PII Metals, 1 ml/L of Vitamin mix, 2 g/L of  $\text{KH}_2\text{PO}_4$ , 43.8 g/L of glucose and 0.5 ml/L of K60K antifoam. M-6 medium has the same ingredients as the M-3 medium except that it contained 12 g/L of Nutrex 55 (Red Star Specialty Products, Milwaukee, WI), a spray dried form of inactive Bakers yeast, instead of soy flour. The oil samples were purified and analyzed for arachidonic acid (ARA) content. The results of these two fermentations are shown in Table 7 below.

TABLE 7

ARA Production by S12 cells from Large Fermentations				
Vessel	Approx. Biomass Yield	Approx. ARA Yield	Ferm. Time	ARA Prod.
B23	22 g/L	2.3 g/L	65.5 h	0.84 g/L/day
B20	20 g/L	2.3 g/L	65.5 h	0.84 g/L/day

Oil was extracted from the fungal biomass produced in the two fermentations according to the following process. Samples of S12 fermentation broth from vessels B23 and B20 were filtered under vacuum to produce a cake of biomaterial which was isolated and dried in a steam oven. The dried biomaterial (200 g) was extracted with hexane (2 x 600 ml) in a waring blender to simulate a wet milling process. The milled fungal biomass was filtered to remove solids and the hexane was evaporated to afford a crude oil. Approximately 75% of the theoretical oil content was recovered in this wet milling process. The crude oil was purified by passing through a column of silica gel and the neutral oil fraction (triacylglycerides) was isolated by eluting the column with 30% ethanol:acetic acid in hexane. Fractions containing neutral oil (90% of crude oil) were pooled and concentrated to give a pure oil fraction which was analyzed by gas liquid chromatography. The results of the fatty acid analyses performed on the purified oil samples are shown in Table 8 below.

TABLE 8

Fatty Acid Content of S12 Oil		
Fatty Acid	% FATTY ACID	
	S-12 (B20)	S-12 (B23)
C16:0 Palmitate	10	11
C18:0 Stearate	12	12
C18:1 n-9 Oleate	14	16
C18:2 n-6 Linoleate	10	9
C18:3 n-6 GLA	3	3
C20:0 Arachidate	1	1
C20:3 n-6 Homo GLA	3	3
<b>C20:4 n-6 Arachidonic Acid</b>	<b>41</b>	<b>37</b>
C22:0 Behenate	2	2
C24:0 Lignocerate	4	4

The results indicate that the purified oil obtained from biomass produced in vessel B20 contained 41% arachidonic acid and the oil from the biomass produced in vessel B23 contained 37% arachidonic acid.

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims:

#### Claims

1. A method of producing arachidonic acid, comprising culturing micro organisms of the genus *Mortierella* sect. *schmuckeri* in a medium comprising a source of assimilable nitrogen.
2. A food product comprising a micro organism of the genus *Mortierella* sect. *schmuckeri* and a food material.

3. A food product comprising lipids recovered from a micro organism of the genus *Mortierella* sect. *schmuckeri* and a food material.
4. A therapeutic agent comprising lipids recovered from a micro organism of the genus *Mortierella* sect. *schmuckeri*.
5. The inventions of claims 1, 2, 3 or 4 wherein said *Mortierella* sect. *schmuckeri* is capable of producing at least about 0.70 grams of arachidonic acid per litre per day.
6. The inventions of claims 1, 2, 3 or 4 wherein said *Mortierella* sect. *schmuckeri* is of a species selected from the group consisting of *Mortierella schmuckeri* and *Mortierella camargensis*.
7. The inventions of claims 1, 2, 3 or 4 wherein said *Mortierella* sect. *schmuckeri* is capable of growing as a dispersed filamentous form when grown under liquid culture conditions.
8. The method of claim 1 wherein said method further comprises recovering lipids comprising said arachidonic acid from said *Mortierella* sect. *schmuckeri*.
9. The food products of claims 2 or 3 wherein said food material comprises an animal food.
10. The food products of claims 2 or 3 wherein said food product has a total fatty acid content in which up to about 20% by weight of total fatty acids is arachidonic acid.
11. The food product of claim 3 wherein said food material comprises an infant food material.
12. The method of claim 1 wherein said medium comprises a complex nitrogen source which increases arachidonic acid production by said *Mortierella* sect. *schmuckeri* by at least about 50%, as measured per cent cell dry weight or per cent of total fatty acids in an oil, compared with *Mortierella* sect. *schmuckeri* grown in the absence of said complex nitrogen source.

# **EXHIBIT F**



US005773075A

**United States Patent** [19][11] **Patent Number:** **5,773,075****Todd**[45] **Date of Patent:** **Jun. 30, 1998**

[54] **HIGH TEMPERATURE COUNTERCURRENT SOLVENT EXTRACTION OF CAPSICUM SOLIDS**

[75] Inventor: **George N. Todd**, Kalamazoo, Mich.

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[21] Appl. No.: **766,504**

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[51] Int. Cl.<sup>6</sup> ..... **A23L 1/221**

[52] U.S. Cl. .... **426/638; 426/651; 426/655; 426/425; 426/429**

[58] Field of Search ..... **426/638, 650, 426/651, 655, 425, 428, 429, 430**

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[57] **ABSTRACT**

Principal components of paprika, red pepper, pungent chili, or other plants of the genus *Capsicum* containing carotenoid pigments are simultaneously extracted and concentrated with an edible solvent in a series of mixing and high temperature and pressure mechanical pressing steps using edible solvent and a countercurrent extraction procedure. The extract containing the carotenoid pigments may be hydrated and then centrifuged to remove fine particulate solids and gums. A solution having several times the concentration of the carotenoid pigments and other flavor and aroma components of the starting raw material is obtained. The residual press solids may be cooled and hydrated following the last pressing operation. The residual press solids and extract have significantly reduced bacterial counts as a result of the temperatures, high pressure, and high shear utilized, as well as the low moisture levels employed, thus producing not only a food grade extract but also a food grade residual solid having low bacterial counts and predictable, standardized levels of the principal components of interest. The degree of browning or caramelization of the residual press solids is controlled, and the resistance to oxidative deterioration of the carotenoid pigments of both the extract and the residual solid is improved, by control of the temperature employed. Additionally, edible antioxidants can be included in the solvent to enhance the stability of both the extract and the residual solid.

**18 Claims, 2 Drawing Sheets**

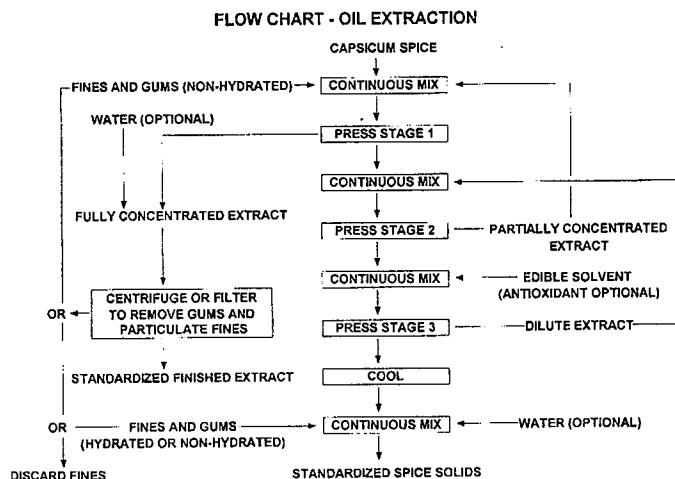
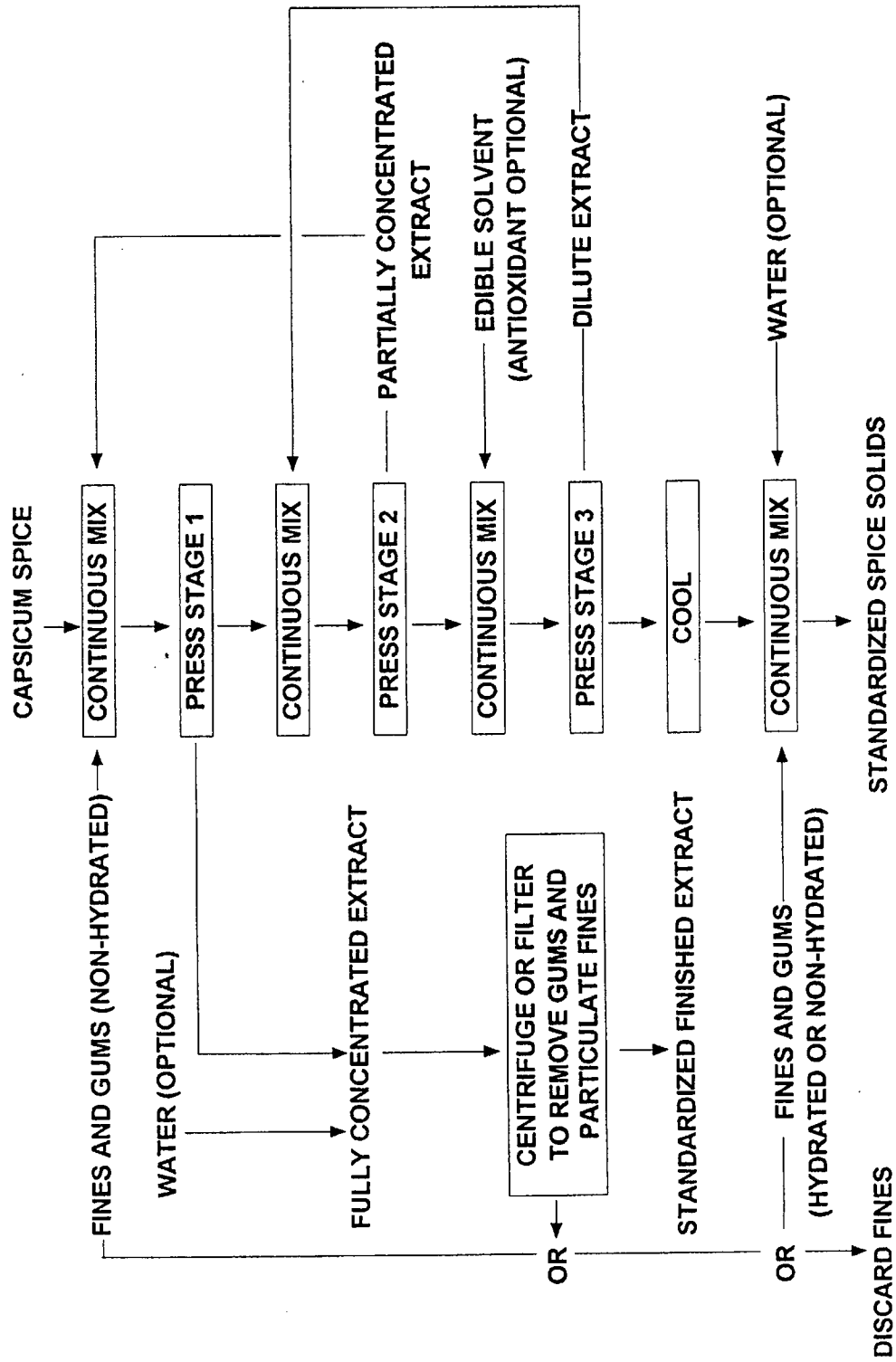
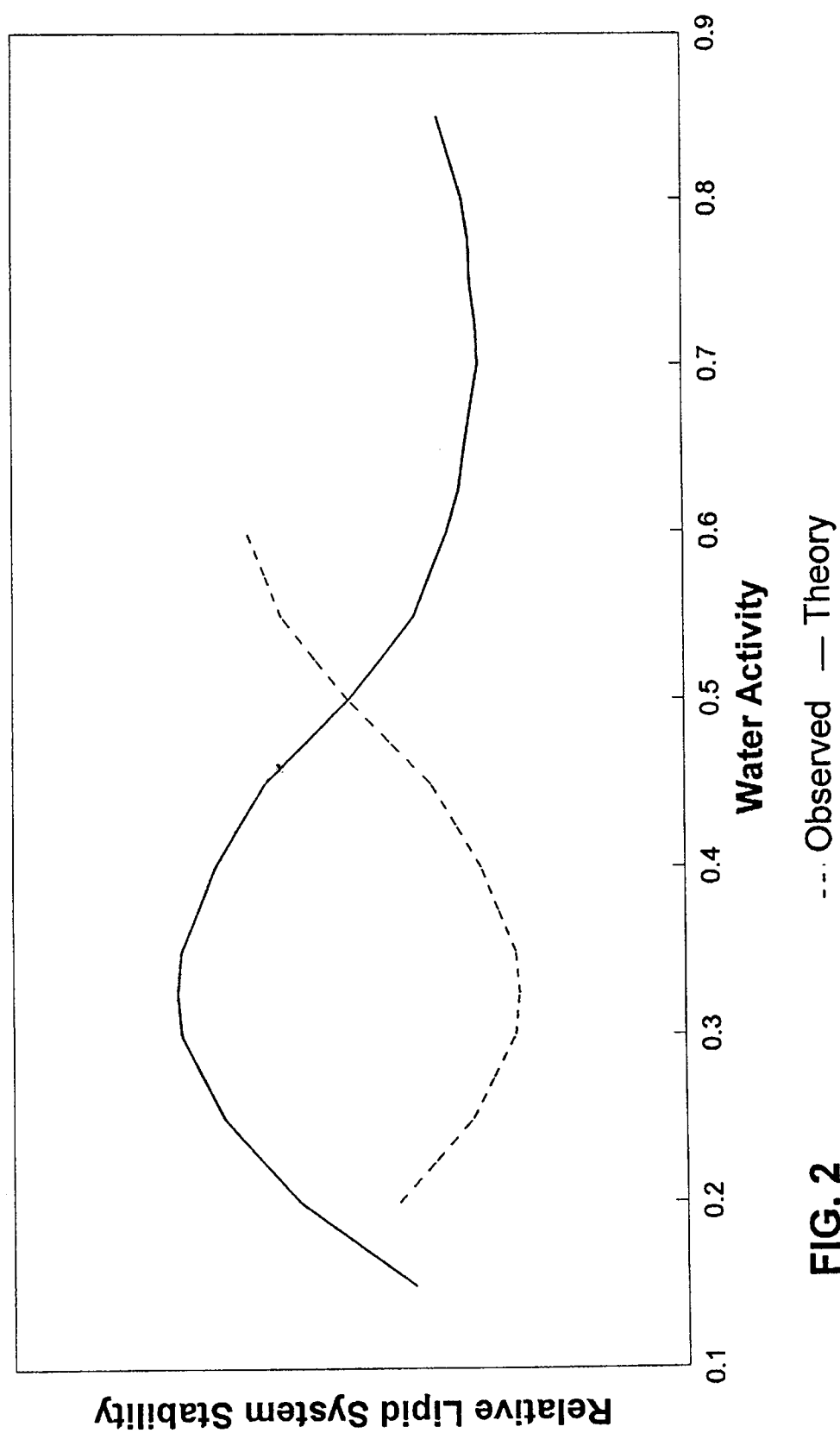


FIG. 1

## FLOW CHART - OIL EXTRACTION



**FIG. 2**

## HIGH TEMPERATURE COUNTERCURRENT SOLVENT EXTRACTION OF CAPSICUM SOLIDS

### FIELD OF THE INVENTION

The extraction of the principal significant components of spice plants of the genus *Capsicum*, representatively paprika, red pepper, and chili, containing carotenoid pigments using edible, food-grade solvent.

### BACKGROUND OF THE INVENTION AND PRIOR ART

The present invention relates to a method of increasing the color stability and reducing the microbial counts of both oleoresin *Capsicum* and the residual cake from which the oleoresin has been extracted. The process simultaneously extracts and concentrates the principal flavor, aroma, color, and other active compounds of the genus *Capsicum*, produces a concentrated and standardized food-grade extract of active components, and a standardized food-grade residual solid, with both the extract and residual solids having improved resistance to color loss and having significantly reduced microbial counts.

Concentrated extracts of the genus *Capsicum* are universally used for flavoring and coloring of food, beverages, and pharmaceuticals. These extracts are traditionally used where a standardized, sterile, and uniform concentrate offers the benefits of control which are inherently difficult to obtain from raw spice, or where the bulk of the raw material is not needed or undesirable.

Ground *Capsicum* solids are universally used for flavoring, coloring, and imparting otherwise favorable characteristics to food and beverages where the bulk, functional characteristics, and appearance of the food or beverage is important.

Traditional extraction processes for the manufacture of concentrated extracts (concentrated several fold as compared with the raw material) involve not only the use of various non-edible solvent systems, but also a large proportion of solvent in relation to the compounds of interest. Many require the use of petroleum distillates, chlorinated solvents, or highly flammable solvents which must be eliminated almost completely from the finished products to make them safe for consumption. These systems require expensive distillation equipment and special precautions must be taken to ensure worker safety and to limit environmental impact. The intensive processing required often destroys, modifies, or loses some of the more unstable compounds, delicate aromas, flavors, or pigments. More significantly, the last traces of undesirable non-edible solvents are very difficult to separate from the concentrated extract. The residual solid must necessarily contain the same residual non-edible solvents, which are removed only with difficulty. Such residual solvents limit the potential use of the residual solid for human consumption, and are potential environmental contaminants.

Other concentration techniques rely on high pressure equipment to obtain good solvating properties from gases, e.g., liquid or supercritical CO<sub>2</sub> (U.S. Pat. No. 4,490,398). High pressure liquefied or supercritical gas extraction requires expensive equipment and has limited solvating abilities for some compounds requiring the addition of cosolvents, or solvents such as propane and butane, which are also difficult to control and may be environmentally sensitive or undesirable in a finished product.

Following extraction and desolventization, the concentrated extract is often standardized with edible solvents and

emulsifiers to provide a concentrate with reproducible levels of the active or principal compounds of interest to the user.

In an effort to overcome the shortcomings and risks associated with the above-mentioned processes, extraction has been carried out using edible solvents such as vegetable oils or lard. Typical extraction procedures are disclosed in U.S. Pat. Nos. 3,732,111; 2,571,867; and 2,571,948. These methods require a relatively large volume of solvent in relation to the compounds of interest and result in a dilute extract which is limited in its application and which has few of the advantages of the concentrates which can be produced using volatile solvents.

U.S. Pat. No. 4,681,769 discloses a method for simultaneously extracting and concentrating in a series of high pressure countercurrent mechanical presses using relatively small amounts of vegetable oil as the solvent in an attempt to overcome the problem of dilution inherent in earlier processes. This method suffers from severe limitations in temperature and pressure ranges in an attempt to avoid unacceptable oxidative damage, color loss, yield losses, and flavor changes with the final result being that contact times must be unduly extended for up to 16-24 hours, adding greatly to the cost of the process. Extraction cycle times are unduly long for a given size pressing operation, and the process does not provide for a controlled degree of browning or for sterilization of the extract or of the residual solid. It is also limited to temperatures of less than 100° F. and thus it does not allow for the use of edible solvents which have a melting point of more than 100° F. or which are highly viscous at temperatures of less than 100° F. Maximum pressures of up to about 500 PSI (cone pressure) are claimed and this severely limits the efficiency and throughput rate for a given size pressing operation, as shown by the disclosure of this patent.

Traditional methods for the sterilization of ground spices, including *Capsicum*, involve the use of extremely toxic substances such as ethylene oxide or methyl bromide, irradiation, or steam and moisture treatment to reduce plate counts to less than 100,000. Chemical sterilization and irradiation of spices are disagreeable to the consumer because of the perceived risk of residual chemicals and/or radiation remaining in the plant matter and, as a result, several processes using added moisture, such as water or steam, at elevated pressures have been developed as alternatives. Typical sterilization procedures are disclosed in U.S. Pat. Nos. 4,210,678, 4,790,995, and 4,910,027. All sterilization processes are inherently costly in that they require a separate processing step or steps to accomplish the sterilization, and also present the possibility of further degrading the more unstable components. Addition of moisture or water vapor, as disclosed in U.S. Pat. Nos. 4,210,678 and 4,910,027, prior to or during the heating and sterilization process results in a cooked aroma not typical of the fresh, dehydrated spice and also results in steam distillation and loss of some of the volatile flavor and aroma constituents.

U.S. Pat. Nos. 4,790,995 and 4,910,027 require the addition of a coating of animal protein to protect the spice from the loss of volatile aroma compounds during the sterilization process with water vapor. U.S. Pat. No. 4,210,678 requires bringing the moisture of the spice to above 8-14%, in some cases up to 16-20%, and holding the spice for an extended period of time prior to sterilization to equilibrate the moisture. This additional step is costly and time consuming. In the case of *Capsicum*s, severe browning and off aromas and flavors are developed in the presence of moistures in excess of 8% at elevated temperatures above 180° F.

Traditional methods for controlling the browning or degree of caramelization of *Capsicum* solid to enhance its



visual appearance involve the use of elevated temperatures and the addition of vegetable or animal fats or oils to bring up the surface color and luster of the ground spice. This requires a separate and costly processing operation.

Above all, there is the unsolved problem of obtaining satisfactory yields, quality, and throughput rates of acceptable extract having an acceptable content of active principle in the edible solvent without undesirable oxidative damage to, and reduced stability of, the principal compounds of interest, while at the same time providing for simultaneous sterilization of both the spice solid and extract.

Obviously, existing prior art procedures leave much to be desired, and it is a primary objective of the present invention to provide a procedure for the production of Capsicum products having enhanced color stability and which otherwise obviates the shortcomings of the prior art.

#### OBJECTS OF THE INVENTION

Accordingly, it is an object of the present invention to provide a process for simultaneously and rapidly extracting and concentrating the principal components of Capsicum solids, at temperatures of at least 130° F., preferably 130° to about 450° F., in a process which is completely free of petroleum, chlorinated or highly flammable solvent, does not require high pressure gas handling equipment, does not require distillation for solvent removal, uses only food-grade edible solvents which are typically used in the trade to standardize the resulting extract to a desired concentration, and provides a product which is free of adulterants and impurities.

Another object of this invention is to prepare such a concentrated extract by a process which is simple, environmentally friendly, and economical.

A further object of this invention is to prepare a residual solid or press cake which is edible, free of residual petroleum distillates, chlorinated solvent, or other adulterants, which is standardized with respect to the principal components of commercial interest, which has a predictable and controlled degree of brownness or caramelization, and which has a controlled level of water activity with its attendant increased resistance to oxidative deterioration of the carotenoid pigments and color loss.

A still further object of this invention is to provide such a process wherein antioxidants can be added to the edible solvent system so as to protect the concentrated extract and the residual solids against oxidative degradation of the principal components of interest, i.e., flavor, aroma, and color, which are extracted from the raw plant material or left in the residual solids.

Still a further object of this invention is to prepare an edible extract and an edible residual solid with reduced microbial activity by a process wherein the moisture of the Capsicum spice is kept below 8%, thereby avoiding the loss of volatile flavor and aroma constituents and avoiding the development of uncontrolled browning and off flavor development at temperatures in excess of 130° F. which are necessary to effect high extraction efficiencies, reduction in microbial activity, and improved stability of the carotenoid pigments in both the extract and in the residual solids.

Yet a further object of this invention is to prepare an extract with increased resistance to oxidative degradation of the carotenoid pigments and consequent color loss.

Other objects will be apparent to one skilled in the art to which this invention pertains and still others will become apparent hereinafter as the description proceeds.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates the process of the present invention, including the several process steps involved in the simultaneous extraction and concentration of Capsicum solids, to produce the desired extract and sterilized residual solid, both of which have increased resistance to oxidative degradation, and which can be readily standardized to desired levels of the principal components of interest. Although the process illustrated comprises three extraction stages, the number of stages can be decreased to two or increased to more than three to effect the desired relative principal component concentration in the extract and in the residual solid.

FIG. 2 illustrates the relationship between the water activity and relative lipid stability of food systems as incorrectly described in current literature and as currently found.

#### SUMMARY OF THE INVENTION

The invention, then, inter alia, comprises the following, alone or in combination:

A continuous multistage mixing, high pressure pressing, and countercurrent extraction process for the production of a concentrated edible extract and edible residual solids, both of reduced bacterial content, with the extract having enhanced resistance to oxidative deterioration of carotenoid pigments therein, and both of which contain carotenoid pigments, flavor, and aroma, from plant material solids of the genus Capsicum, comprising the following steps:

- subjecting said Capsicum solids to a countercurrent extraction process involving a plurality of mixing and pressing stages, including first and last mixing stages and first and last pressing stages, together with five to about fifty percent by weight of an edible solvent, to produce an extract and residual solids,
- continuously returning the extract from each pressing stage to the previous mixing stage, and finally
- separating the extract from the first pressing stage and separating the residual solids from the last pressing stage, all pressing stages being carried out at a temperature of at least 130° F.; such
- a process, wherein the temperature is 130° to about 450° F.; such
- a process, wherein the Capsicum solids are subjected to internal pressures in the press stages of at least 6,000 pounds per square inch; such
- a process, wherein the weight of the edible solvent is 5% to about 20% by weight of the Capsicum solids; such
- a process, wherein the moisture content of the starting Capsicum solids is less than 6% by weight, and wherein bacterial count reduction is effected at this low moisture content, thereby avoiding undesirable loss of volatile flavor and aroma constituents and avoiding the development of cooked, off flavors and aromas which occur at higher moisture contents; such
- a process, wherein the Capsicum solids extracted in the process are selected from the group consisting of paprika, red pepper, and chili; such
- a process, wherein the edible solvent is selected from the group consisting of soybean oil, corn oil, cottonseed oil, rapeseed oil, peanut oil, mono-, di-, or triglycerides, lecithin, edible essential oils, sesame oil, edible alcohols, hydrogenated or partially hydrogenated fats or oils, polyoxyethylene sorbitan esters, limonene, edible animal fats or oils, mixtures thereof, and edible derivatives thereof; such

- a process, wherein fine particulate solids are filtered or centrifuged from the extract and alternatively discarded, returned to a mixing or pressing stage of the process, or incorporated in the final residual solids; such
  - a process which includes the steps of hydrating the final extract to add water to the extent of 5% to 200% by weight of the gums and fine particulate solids therein and filtering or centrifuging to remove said gums and solids; such
  - a process including the step of returning separated hydrated gums and solids to the final residual solids; such
  - a process, including the step of rehydrating the final residual solids with water to a water activity greater than 0.3  $A_w$ , for color stabilization thereof; such
  - a process, wherein the solids are rehydrated to a water activity of about 0.4 to 0.6  $A_w$ ; such
  - a process, wherein an effective color-stabilizing amount of an edible antioxidant or chelator is included in the edible solvent; such
  - a process, wherein the antioxidant comprises an antioxidant selected from the group consisting of lecithin, ascorbic acid, citric acid, tocopherol, ethoxyquin, BHA, BHT, TBHQ, tea catechins, sesame, and the antioxidant activity from an herb of the Labiatae family; such
  - a process, wherein the antioxidant comprises a naturally-occurring antioxidant from an herb of the family Labiatae or powdered ascorbic acid; such
  - a process, wherein the antioxidant comprises the antioxidant activity from an herb selected from the group consisting of rosemary, thyme, and sage, and such
  - a process, wherein the temperature is above 180° F., and preferably between about 180° F. and about 235° F.
- Moreover, an extract of plant solids of the genus *Capsicum* produced by the process having increased color stability due to the high temperature employed in its production; such
- an extract of plant solids of the genus *Capsicum* produced by the process having a high color value and a low bacterial count due to the high temperature employed in its production and due to a low water content of less than 6% in the starting *Capsicum* solids; and
  - rehydrated cake solids produced by extraction of *Capsicum* solids according to the process having a high degree of color stability due to the high temperature employed in production thereof and due to the level of water activity  $A_w$  present therein; and
  - an extract of a plant of the genus *Capsicum* produced by the process in the form of a clear solution with gums and particulate solids therein converted to their insoluble hydrates and then removed from the extract; and
  - a hydrated extract of a plant of the genus *Capsicum* produced in the process and having gums and particulate solids therein in their insoluble hydrated form; and finally
  - an extract of plant solids of the genus *Capsicum* having improved color stability produced according to the process due to an edible antioxidant therein.

#### THE PRESENT INVENTION

##### IN GENERAL

Raw *Capsicum* spice solids, either ground (usually to pass US 40 mesh, and preferably to pass at least US 20 mesh) or

unground if coarse particles are desired in the residual solid or cake, e.g., *Capsicum* solids having a moisture range of about 0.5% to 16% by weight, preferably 0.5 to 8%, and most preferably 1.5% to 6% by weight (ASTA method 2.0), are subjected to a mixing stage, preferably high shear, and in at least one stage an edible solvent is thoroughly dispersed throughout the raw plant material solids.

Typical *Capsicum* starting plant materials include, for example but without limitation, the dried ripe fruits of *Capsicum frutescens* L. (chilies), *Capsicum annuum* L. (Spanish peppers), *Capsicum annuum* L. var. *longum* Sendt, its hybrid Louisiana Sport Pepper, and *Capsicum chinense* (Scotch Bonnet or habenero), all by way of example and not by way of limitation.

The comminuted or uncomminuted plant material is subjected to a plurality of mechanical pressing stages, whereby a concentrated extract of principal components is obtained and a final utilizable and preferably standardized residual solid is produced. The selected edible solvent is introduced into the residual solid at a mixing stage at some point prior to the last pressing stage. The edible solvent, now containing extract, is cycled back to the previous stage, thus always supplying a solvent extract with increasing principal component concentration to the previous mixing and pressing stages. As the extract/edible solvent is passed through each stage countercurrent to the solids flow, a portion of the edible solvent is squeezed or pressed out, thereby extracting a portion of the principal components of interest. As the edible solvent/extract passes countercurrent to the solids, the extracted principal components are progressively concentrated in the extract in a continuous process and the residual principal components end up in the final residual solids known as the cake.

By varying the pressure, temperature, spice solids feed rate, solvent addition rate, and the number of mixing and pressing stages, the concentration of the principal components can be controlled in both the extract and the residual solid.

As will be apparent to one skilled in the art, variations in the process of the present invention can be employed to produce variations in result, the most advantageous of which are the production of both plant material extract of marketable potency and edible residual solid plant material also characterized by marketable potency. For example, using 200 ASTA paprika starting material of about 5% moisture, a 20% soy oil addition, and leaving a residual cake extractable yield of 9.8% by weight of the starting plant solids material, gives an extract with a color value of 850 ASTA and a residual cake color value of approximately 50 ASTA. Contrastingly, using a 10% soy oil addition (instead of 20%) yields a cake having about 65 ASTA color value and, by increasing the residual cake extractable yield to 12.5% by weight of starting plant solids material, the color value of the residual cake rises to about 100 ASTA and that of the extract to about 1400 ASTA. The lowest color extract for paprika normally traded is 1,000 ASTA.

Although less than 20% edible oil addition is highly desirable and can be used in many cases, with some edible solvent systems wherein the principal compounds of interest have a limited solubility, or when a more dilute extract and/or lower concentration of principal compounds is desired in the residual solids, more than 20% by weight of edible solvent addition will be required inasmuch as a suitable concentration of principal compounds in the finished extract and in residual solid can in some cases be produced only by the employment of the higher dilution.

Due to the successive treatments of high pressure and pressure relief, with pressures ranging from 6,000 to 30,000

PSI in the pressing stages of the operation, in the presence of added edible solvent, e.g., vegetable oil, and due to frictional heat generated in these high pressure zones, both the residual solid and the extract exiting the process surprisingly have a significantly reduced microbial load over that of the starting material even at moisture levels significantly lower than those indicated by the prior art and, also surprisingly, exhibit increased resistance to oxidative degradation of the carotenoid pigments which are responsible for the characteristic red-yellow color of Capsicums.

The extract from the first or any selected pressing stage may be centrifuged or filtered to provide the finished extract free of particulate solids. Preferably, the fine particulate solids and gums in the extract may be hydrated to about 5% to 200% by weight of the gums and solids prior to centrifugation or filtration to give a crystal clear extract. If water is not used to hydrate the solids and gums, the fine particulate solids from the extract may conveniently be combined with the final residual solids, recycled back into mixing and pressing stages of the process, or alternatively discarded. If water is used to hydrate the solids and gums, it is preferred that the solids and gums be added back to the final residual press solids or discarded.

The edible solvent employed according to the process of the present invention, as illustrated by the following Examples, may be any edible solvent and especially those selected from the group consisting of soybean oil, corn oil, cottonseed oil, rapeseed oil, sesame oil, peanut oil, mon-, di-, and triglycerides, lecithin, essential oils of spices, herbs, or other plants, edible alcohols, propylene glycol, glycerine, hydrogenated or partially hydrogenated fats or oils, limonene, polyoxyethylene sorbitan esters, or any other edible vegetable or animal fat or oil, or mixture thereof, or edible derivatives thereof, the essential aspects of the solvent being that it serves as an extraction aid in which the principal components of the material being extracted are soluble and that it be edible.

The edible solvent, according to the present invention, is combined with the raw material solids to be processed in a proportion of about 5% to about 50% by weight, and frequently amounts as low as 5 to 20% by weight are possible, based on the weight of the starting raw material solids to be extracted. The lower percentages frequently produce a more acceptable and marketable concentration of principal components of interest in both the extract and the residual solids.

The temperature to be employed during the processing and especially in the pressing stages of the process of the invention may be varied widely, but the process is generally carried out at a temperature below about 450° F., and between about 130° F. and 325° F., most preferably above 180° F. and especially between about 180° F. and 235° F.

Temperatures in excess of 130° F. are advantageously employed to achieve acceptable yields and increased throughput rates as compared to the prior art. Higher temperatures are employed to control an increased degree of browning and, most importantly, to reduce the microbial load of both the solids and the extract while at the same time imparting increased resistance to oxidative degradation of the carotenoid pigments in both the extract and the residual solids. Thus, when it is desired that the residual solids from the process have a desirable darkened, caramelized appearance and/or flavor, a reduced microbial load, and increased resistance to oxidation, this is readily attained by increasing the temperature of the solids and the extract during the process, especially during the pressing stages thereof.

When an antioxidant or chelator is introduced into the process for protection of the spice being processed, this is

preferably another plant material or an extract thereof, preferably of the Labiatae family, such as rosemary, thyme, or sage, which is known for its protective antioxidant activity (U.S. Pat. No. 5,209,870), or sesame, or tea catechins, but may alternatively be a suitable edible and preferably an approved food grade additive such as ethoxyquin, BHA, BHT, TBHQ, tocopherol, Vitamin C (e.g., as in U.S. Pat. Nos. 5,290,481, 5,296,249, or 5,314,686), citric acid, EDTA, or the like. The process of the present invention is particularly adaptable to the extraction of any Capsicum plant material solids containing carotenoid pigments or other components which provide color and/or flavor, pungency, or aroma to a food with which combined.

## DETAILED DESCRIPTION OF THE INVENTION

The following examples are given to illustrate the present invention but are not to be construed as limiting.

### EXAMPLE 1

#### Paprika Extraction

Dehydrated paprika (5.5% moisture) is ground in a hammer mill and the resulting ground paprika (95% passing US 40 mesh) is admixed with about 10% by weight of soy bean oil and processed in a countercurrent extraction system involving three (3) pressing stages, each using an Egon Keller Model KEK-100 Screw Press, with the extracts from the second and third stages being returned to the preceding mixing stage before being removed from the process at the end of the first press stage. A high shear, high speed pin mixer or equivalent is used to mix the soy oil or extracts from the second and third press stages into the ground spice or residual solid from the preceding stage. This recycling is continuous. The raw material paprika solids are continuously fed at a rate of about 240 lbs. per hour with a total contact time in each mixing stage of about 15-60 seconds. The residence time in each press is 5-60 seconds. The pressing stages are operated at about 10,000 PSI internal pressure and about 200 degrees Fahrenheit, which is maintained by cooling with water through the bore of the press shafts. The starting color value of the ground paprika solids is 200 ASTA. The principal components extracted and standardized in both the extract and the residual solid are the carotenoid pigments. The resulting final soy-paprika extract has a color value of about 1,375 ASTA and the reground paprika residual solid from the final (3rd) press stage has a color value of about 85 ASTA.

### EXAMPLE 1A

#### Variation

By varying the percentage of edible solvent employed from about 5% to 20%, the pressure from about 6,000 to 30,000 PSI, the number of countercurrent mixing and pressing stages from 2 to 5, with return of the extract from each press stage to the preceding mix stage before final removal from the process in the first press stage, varying the temperature from about 130° F. to 280° F., and removing the seed from the paprika solids prior to grinding, the resulting extract ranges in color value from about 2,700 ASTA to about 800 ASTA and the residual solids range in color value from 180 ASTA to 35 ASTA.

By regrinding the residual solids (from the final stage) just as is done with fresh, dehydrated paprika, a product in every way comparable to commercially available ground paprika solids is produced. After filtering or centrifuging off the fine particulate solids, the extract can be directly substituted for commercially available paprika oleoresin in every respect.

By varying the pressing temperature of the process from about 130° F. to 325° F., the hue of the reground residual solid is varied from slightly browned to a dark chocolate brown, demonstrating that the degree of brownness can be controlled by the pressing temperature employed. The degree of "brownness" is measured using a Hunter Labscan Spectrocolorimeter with 0 degree illumination, 45 degree circumferential viewing, illuminant D65, 10 degree observer, Ceilab coordinate system. The hue of the paprika powder is measured by placing the powder in a 2.5-inch diameter cuvette, shaking gently to ensure even coverage, and measuring through the bottom of the cuvette. The results of the varied operating temperatures of the process are shown in Table I. The designation L\* is indicative of the "lightness" of the sample with the higher numbers being lighter or less browned, and the lower numbers being darker or more browned.

TABLE I

Processing Temperature	Visual Appearance	L* Values
130° F.	Red	40.18
150° F.	Tan-Red	37.25
200° F.	Light Brown Red	33.22
280° F.	Dark Brown Red	29.16
325° F.	Chocolate Red	22.85

The data clearly demonstrate that the degree of browning can be controlled by varying the press temperature at which the process is conducted. This broadens the applications or uses of the residual solid to include a base for toasted chili powder and as a replacement for browned, caramelized paprika. The residual solid can be substituted for ground paprika or chili powder in many common applications and a separate processing step for browning to a desired degree is not required.

The starting ground paprika solids have an aerobic plate count (Analysis run according to Bacterial Analytical Manual By AOAC, 8th edition, 1995, and ISO-GRID Methods Manual, 3rd edition, 1989) of about 14,000,000. The residual solids exiting the extraction system have a count of about 2,000 to 200,000, with the lower count being achieved at the higher temperatures. This is a significant reduction and makes the residual solids per se suitable for any application where treatment with ethylene oxide or irradiation would normally be required.

## EXAMPLE 1B

## Antioxidant Addition

The foregoing example is repeated with all materials and conditions being the same, except that the soybean oil edible solvent is supplemented with an antioxidant blend at a concentration of 3% by weight of the original ground paprika solids. The blend consists of about 29% lecithin, 20% powdered ascorbic acid, 5% citric acid, 15% tocopherol, and 1% rosemary extract (in accordance with Chang and Wu U.S. Pat. No. 5,077,069).

The stability of (1) the resulting extract and (2) the residual solids is compared in each case with an untreated control. In such evaluation, the paprika extracts are plated on flour salt to an extent of 2.4% by weight with a mortar and pestle. Two-gram samples are weighed into 13x100 mm test tubes. The test tubes are stored in a thermostatically-controlled oven at 65° C. Samples are withdrawn periodically, extracted with acetone, and the color at 460 nm of a standard (%) dilution in acetone is determined spectrophotometrically. In the evaluation of the residual solids, two-gram samples of the reground residual solid are substituted for the flour salt dispersions.

The procedure for the "standard dilution" is as follows: The initial color of the dispersion is determined by pouring two grams of the original dispersion into a 100-ml flask. Acetone is added up to the 100-ml level. The flask is inverted several times. The flour salt is allowed to settle for five minutes. Then three ml of the dilution is pipetted into a 25-ml flask and diluted up to the 25-ml level. The absorbance is read at 460 nm. The 460 nm color is determined by the formula:

$$460 \text{ nm color} = \frac{(\text{absorbance at } 460/12)}{(\% \text{ dispersion}/100)}$$

where the percent dispersion is determined by the formula:

$$\% \text{ dispersion} = \frac{\text{color units on the flour at } 460 \text{ nm}}{\text{color of the test sample at } 460 \text{ nm}}$$

to translate to ASTA color, multiply the 460 nm color by 820.

The color is plotted against time and the time for 1/3 of the starting color to fade is reported as the 1/3 life. This is a highly-reproducible measurement, which is sufficiently accurate to evaluate the effectiveness of the antioxidants and will assist the practitioner to optimize formulations for specific uses.

The final extract from the first press stage of the unprotected or unstabilized process has a color value of about 1375 ASTA and a 1/3 life of 6.5 hours as compared to a color value of about 1600 ASTA and a 1/3 life of 63 hours for the extract from the protected material. The color value of the unprotected or unstabilized residual solids is about 85 ASTA with a 1/3 life of 54 hours, compared to the protected solids which have a color value of about 95 ASTA and a 1/3 life of 155 hours. This clearly demonstrates that inclusion of antioxidants can improve not only the color yields from the extraction process but also at the same time improve the color stability of both the extract and the residual solids.

Other suitable antioxidants (e.g., lecithin, ethoxyquin, butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), tertiary butyl hydroxy quinone (TBHQ), sesame, tea catechins, and Labiatae herb antioxidant activity, finely-divided ascorbic acid, tocopherol, citric acid) can be substituted in whole or in part for the specific antioxidant mixture employed with similar desirable color-protective results, preferably a naturally-occurring antioxidant from an herb of Labiatae family, e.g., rosemary, sage, or thyme, or powdered ascorbic acid.

## EXAMPLE 2

## Effect of varying operating temperatures

Dehydrated paprika solids (2.5% moisture) were ground in a hammer mill and the resulting ground paprika (95% passing US 40 mesh) was processed with about 15% by weight of soy bean oil in a countercurrent extraction system as in Example 1 involving two (2) pressing stages, with extracts from the second press stage being returned to the preceding (first) mix stage before being removed from the process at the first press stage.

Upon exiting the first press stage, distilled water was metered continuously into the crude extract at a rate of 75% by weight of the gums and solids by means of an inline static mixer. The weight of the gums and fine particulate solids in the extract was determined by diluting one gram of the crude extract in nine grams of acetone. The mixture was spun down for three minutes at 2000 G's in a laboratory centrifuge. The solids separated were air dried and the weight of the gums and solids was calculated as a percentage of the

weight of the starting extract. The hydrated gums and solids removed from the extract were continuously returned to the final residual press solids via a high shear, continuous pin mixer installed immediately following a water-jacketed cooling screw which received the residual solids from the second press stage.

Prior to hydration and centrifugation, the extract contained approximately 10% by weight of gums and fine particulate solids as determined by the above-described method. Following hydration and centrifugation the gums and particulate solids amounted to no more than 1% by weight of the extract and the extract was a crystal clear solution, free of any suspended insoluble materials.

The color value of the starting ground paprika was about 150 ASTA. The pressing stages were operated at about 20,000 to 30,000 PSI. The extraction process was started with the presses operating at about 80° F. as measured by the temperature of the cake exiting the presses. The temperature of the presses was controlled by the rate of flow of cooling water through the bore of the press shafts and the screen cages to keep the operating temperatures in the range of 80° to 180° F. Over the time of the extraction run, the operating temperatures of the presses, as measured by the temperature of the cake exiting the presses, was gradually increased to about 255° F. by first slowing and then stopping the flow of cooling water to obtain operating temperatures of 180°–200° F., and then by substituting steam for the water in the shaft and cages at gradually increasing pressures to achieve temperatures of 200°–255° F. Samples of the extracted oil and press residual solids were pulled at various temperature intervals as the temperatures were increased. Samples of the residual solids were pulled at two points, the first (non-rehydrated) immediately after exiting the cake-cooling screw following the final (second) pressing stage, and the second after the thus-cooled residual press solids were rehydrated to a moisture content of about 10%. The samples were assayed for ASTA color, aerobic and anaerobic plate count, and color stability over time using methods employed in Examples 1A and 1B.

The advantages of operating the process at a temperature above 130° F., as indicated by the temperature of the cake exiting the presses, can clearly be seen. The plate count of both the extract and the cake are progressively reduced as the temperatures are increased. (Tables 2 & 3)

TABLE 2

EFFECT OF INCREASING TEMPERATURES ON THE PLATE COUNT OF THE EXTRACT		
Temperature Degree F.	Aerobic Plate Count	Anaerobic Plate Count
80	1,900,000	790,000
130	1,700,000	800,000
150	1,700,000	660,000
170	1,600,000	500,000
175	1,500,000	425,000
180	1,300,000	380,000
190	360,000	150,000
200	300,000	200,000
215	240,000	150,000
225	190,000	65,000
235	170,000	32,000
245	69,000	8,600
255	3,800	830

TABLE 3

EFFECT OF INCREASING TEMPERATURES ON THE PLATE COUNT OF THE PRESS SOLIDS		
Temperature Degree F.	Aerobic Plate Count	Anaerobic Plate Count
80	220,000	55,000
130	160,000	35,000
150	160,000	25,000
170	100,000	20,000
175	32,000	15,000
180	80,000	7,400
190	3,500	800
200	9,800	3,400
215	5,800	2,300
225	4,100	500
235	1,900	1,100
245	5,400	100
255	800	100

The efficiency of extraction is dramatically improved as evidenced by the progressively decreasing ASTA values and the progressively decreasing residual extractable yields of the press residual solids. It is apparent that, to achieve residual extractable yields of less than about 20% by weight of the cake, it is necessary to operate the presses at 130° F. or higher. (Table 4) Moreover, for obvious reasons of efficiency, temperatures above 180° F., and especially between about 180° F. and about 235° F., are greatly preferred.

TABLE 4

PRESS CAKE ASTA AND RESIDUAL YIELDS AT PROGRESSIVELY INCREASING TEMPERATURES		
Temperature Degree F.	Press Solids ASTA	Press Solids Residual Yield
80	87	28.28%
130	76	16.40%
150	65	15.72%
170	61	15.72%
175	53	12.36%
180	43	13.88%
190	42	10.84%
200	44	10.72%
215	41	9.96%
225	39	9.50%
235	33	9.28%
245	32	9.00%
255	35	9.80%

Most importantly, the stability of the extract is not adversely affected and is in fact increased. The results, from Example 2, of an accelerated study on the stability of the extract, generated at varying press operating temperatures can be seen in Table 5. The accelerated study was done according to the procedures described in Example 1B with the colors reported as a percent of the starting color for each respective sample to adjust for the varying color yields at the respective temperatures. These results demonstrate that the extract produced at higher operating temperatures exhibit increased resistance to oxidative color deterioration. This is surprising, as explained in the following.

TABLE 5

PRESS OLEORESIN (EXTRACT) STABILITY, ACCELERATED, 65° C.					
Temperature Degree F.	Hour 2	Hour 4	Hour 8	Hour 12	Hour 17
80	94%	88%	81%	73%	62%
130	94%	89%	82%	75%	64%
170	93%	89%	82%	76%	65%
225	94%	90%	82%	78%	67%
235	94%	90%	82%	77%	69%
255	95%	90%	84%	78%	72%

It is commonly believed that lipid-containing systems, when exposed to heat, will exhibit an increased rate of lipid oxidation that, once initiated, will proceed at an ever-increasing rate. (*Rancidity and its Measurement in Edible Oils and Snack Foods, A Review*, Robards, Kerr, and Patsalides, *Analyst*, February 1988, Vol 113). In fact, prior art (U.S. Pat. No. 4,681,769) claims a process for counter-current, high pressure extraction of Capsicums at less than 100° F. and less than 500 PSI for the express reason of protecting the extracted oil from oxidation.

To confirm the positive effect of high temperature treatment in more controlled conditions, a forty gram sample of hexane-extracted oleoresin paprika, with no diluents added, was heated in a beaker on a heated stir plate at 100° C. for eight and one-half hours. A control sample which was unheated, a sample pulled from the heated beaker after four hours, and a sample of the material heated for the full eight and one-half hours were dispersed on flour salt to make dispersions of 1.2% oleoresin by weight of flour salt. Two gram-portion of the dispersions were weighed into test tubes and placed in a 65° C. oven. An initial ASTA color was run on each dispersion and then ASTA colors were run periodically and the results were plotted versus time to determine the relative stability of the heated and unheated samples. The results are shown in Table 6. It can be readily observed that the heat-treated samples, although they lose some initial color during the heating process, have improved stability over time, thus confirming the improved resistance to oxidation observed in Table 5.

TABLE 6

ASTA VALUES OF HEATED & UNHEATED OLEORESIN PAPRIKA (EXTRACT) OVER TIME			
Hours	Unheated	Heated 4 @ 100° C.	Heated 8 @ 100° C.
0	32.5	31.5	26.0
2	29.0	29.0	25.8
4	26.0	28.0	25.7
6	24.0	27.0	25.5
8	22.5	25.8	25.3
10	21.0	24.5	25.0
12	20.0	23.0	24.8
14	19.0	22.3	24.5
16	18.0	21.8	24.0
18	17.0	21.0	23.5
20	16.0	20.0	23.0
22	15.1	19.0	22.5
24	14.2	18.5	22.1
26	13.4	18.0	21.8
28	12.9	17.5	21.4
30	12.5	17.0	21.0

The non-rehydrated press residual solids produced in Example 2 exhibit decreased resistance to oxidative color loss as the press operating temperatures are increased as predicted by prior art (Bennett et al, U.S. Pat. No. 4,681,769) and as seen in Table 7.

TABLE 7

STABILITY OF NON REHYDRATED PRESS SOLIDS AT VARIOUS PRESS OPERATING TEMPERATURES, EXPRESSED AS PERCENT OF STARTING COLOR RETAINED			
Temperature Degree F.	Week 2	Week 4	Week 6
80	86.7%	82.2%	85.5%
130	89.6%	85.5%	84.6%
170	73.3%	65.3%	58.1%
225	61.7%	35.8%	32.5%
245	68.2%	31.0%	19.3%

But, very importantly, it can be seen that the press residual solids which are rehydrated immediately after exiting the second press stage of the process (Example 2) exhibit significantly increased stability (Table 8) relative to the non-rehydrated solids, thus overcoming the claimed disadvantages from operating at temperatures above 100° F. as set forth in U.S. Pat. No. 4,681,769.

TABLE 8

STABILITY OF REHYDRATED PRESS SOLIDS AT VARIOUS PRESS OPERATING TEMPERATURES, EXPRESSED AS PERCENT OF STARTING COLOR RETAINED			
Temperature Degree F.	Week 2	Week 4	Week 6
80	90%	92%	91%
130	93%	91%	92%
170	92%	92%	91%
225	94%	93%	91%
245	95%	94%	93%

In fact, after discounting for the effect on pigment stability of increasing residual extractable yields in the press solids (Tables 4 & 11) obtained at the lower temperatures, the carotenoid pigments in the residual solids would show enhanced stability for a given residual extractable yield. These are surprising and unexpected results and clearly overcome the supposed obstacle of operating at elevated press temperatures and pressures.

It is further surprising that the color stability of the residual press solids is significantly improved by controlling the water activity ( $A_w$ ) of the solids in ranges above those suggested for the stabilization of lipid-containing systems by extensive studies and particularly by Nelson and Labuza, *Water Activity and Food Polymer Science: Implications of State on Arrhenius and WLF Models in Predicting Shelf Life*, K. A. Nelson & T. P. Labuza, *Journal of Food Engineering* 22, 271-289 (1994). Water activity is defined as the ratio of the vapor pressure of water in a food to the vapor pressure of pure water at the same temperature. Prior art suggests that maximum stability of lipid systems should be attained at water activities of about 0.3 with decreasing stability developing as the water activity is increased above this level. In this example we find precisely the inverse effect on stability of the carotenoid pigments for a given water activity.

In order to confirm the effect of high temperatures in the pressing operation, and to confirm the effect of added moisture, a controlled test was performed on a laboratory scale where the effect of levels of extractable yield in the cake could be controlled to eliminate the effect of variable press cake residual yields on the stability of the carotenoids. A 3,000 gram sample of ground paprika solids (175 ASTA, 9.8% extractable yield) was dried in a lab tray dryer at 100° F. for 16 hours to a moisture content of about 2%. One half of this sample was then heated in an oven at 220° F. for

twenty minutes to approximate the temperature in a pressing operation according to the invention. The other unheated sample served as a control. One hundred gram samples of each of the two materials were rehydrated at approximately 1% intervals up to about 12% moisture. The water activity  $A_w$  of each was determined using a Rotronics Hygroskop DT, model DT2/1-00IV, water activity instrument. Samples were weighed into sealed test tubes, stored at ambient temperatures of about 72° F. in the dark, and the ASTA colors were determined over a period of eighteen weeks to determine the relative rates of color degradation. The color retained (as a percentage of the starting color for each sample to compensate for the effect of color dilution with the rehydration water) was plotted against time.

TABLE 9

PERCENT COLOR RETAINED OF UNHEATED GROUND PAPRIKA AT VARIOUS WATER ACTIVITY RANGES			
Water Activity $A_w$	Week 1	Week 5	Week 18
0.15	74%	57%	42%
0.30	50%	45%	12%
0.40	68%	50%	43%
0.60	83%	68%	55%

TABLE 10

PERCENT COLOR RETAINED OF HEATED GROUND PAPRIKA AT VARIOUS WATER ACTIVITY RANGES			
Water Activity $A_w$	Week 1	Week 5	Week 18
0.15	66%	56%	41%
0.30	60%	50%	45%
0.40	80%	62%	57%
0.60	98%	82%	78%

It can be seen in Tables 9 & 10 that the stability of the carotenoid pigments follows almost precisely the inverse of the curve predicted by Nelson & Labuza (FIG. 2). It can also be seen from these tables that controlled temperature (with concurrent browning) significantly enhances the stability of the carotenoids above a water activity of 0.3 and particularly in the water activity range of 0.4 to 0.6. Water activity ranges higher than 0.6 were not tested as levels marginally higher than this range will support microbial growth which is not acceptable in a dry spice product.

It can be concluded that the stability of the carotenoid pigments found in Capsicums unpredictably does not follow the commonly-accepted and predicted pattern for lipid oxidation with respect to temperature and water activity as suggested in U.S. Pat. No. 4,681,769, or in the cited literature (Nelson and Labuza, *Water Activity and Food Polymer Science: Implications of State on Arrhenius and WLF Models in Predicting Shelf Life*, K. A. Nelson & T. P. Labuza, *Journal of Food Engineering* 22, 271-289 (1994); *Rancidity and its Measurement in Edible Oils and Snack Foods*, A Review, Robards, Kerr, and Patsalides, *Analyst*, February 1988, Vol 113); describing the stability of lipid systems. In fact, high temperature treatment, combined with rehydration of the press solids to a water activity above 0.3, preferably of 0.4 to 0.6, significantly improves stability rather than decreases it. This is a very surprising and unpredicted result.

It is well known that the lipid profile of Capsicum and its extracts, without the addition of any diluents, comprises a mixture of saturated and unsaturated fatty acids, 60-70% being unsaturated linoleic and linolenic, *Lipid and Antioxidant Content of Red Pepper*, Daood, Biacs, et al., Central

Food Research Institute, Budapest, Hungary (1989) and *The Nature of Fatty Acids and Capsanthin Esters in Paprika*, Nawar et al., *Journal of Food Science*, Vol 36 (1971). In fact, Daood et al suggest that "... the presence of triglycerides containing high amounts of unsaturated fatty acids may be an important factor contributing to the fading of paprika during processing and storage." The present findings are just the opposite. Without in any way being limited by theoretical considerations, it is hypothesized that the presently-discovered surprising and unpredicted inverse relationship shown (in Tables 9 & 10) between the stability of carotenoid pigments at given water activities is due to the fatty acids in the substrate being preferentially attacked by the oxidation reaction at the low (from about 0.05 to 0.2  $A_w$ ) and higher water activity ranges (above 0.3, preferably about 0.4 to 0.6  $A_w$ ), thus protecting the carotenoids. At the intermediate water activity ranges (0.2 to 0.4  $A_w$ ), where the lipids are best protected, the carotenoids are more readily and preferentially attacked and exhibit low resistance to oxidative degradation.

Another controlled test was conducted to demonstrate the effect of different extractable yields in the residual solid press cake. The effect of higher amounts of unsaturated fatty acids is evident from the results illustrated in Table 11 where fresh, refined, bleached, and deodorized soybean oil with no antioxidants was added at various percentages based on the weight of the paprika. The color over time was compared to the untreated control in an accelerated study at 65° C. A typical Refined, Bleached, and Deodorized soy oil has a fatty acid composition of 22.3% Oleic (18:1), 51% linoleic (18:2), and 6.8% linolenic (18:3). (*Riegel's Handbook of Industrial Chemistry*, 9th Edition, pg 278). It can be concluded that higher levels of unsaturated fatty acids, such as oleic, linolenic, and linoleic, which are found in most vegetable oils, will improve the color stability of the press residual solids. Levels of extractable yield in the residual solids above about 15-20% by weight of the residual solids is undesirable as the residual Capsicum solids become difficult to handle for most uses and the efficiency of extraction is reduced, i.e., less color can be removed from the spice as the residual yield is allowed to increase by decreasing either the pressure or temperature employed.

TABLE 11

PERCENT COLOR RETAINED WITH VARYING AMOUNTS OF SOY OIL ADDED TO GROUND PAPRIKA				
Percent Addition	Hour 2	Hour 4	Hour 6	Hour 8
0%	65%	59%	52%	50%
5%	90%	83%	74%	72%
10%	92%	84%	75%	74%
15%	94%	87%	80%	78%
20%	96%	91%	83%	81%

It is readily apparent, comparing the results of the controlled test (Tables 9 & 10) on stability of heated vs unheated material, where oil is controlled at a constant level that, at a given added soy oil content in the press residual solids, the color stability of the residual press solids is significantly improved when the Capsicum has been exposed to higher temperatures. This conclusion is not readily apparent in the results shown in Table 8 where the amount of residual vegetable oil left in the press residual solids is higher in the low temperature ranges due to the decreased efficiency of the extraction process at lower temperatures (Table 4). The presence of higher amounts of residual oils there offers some protection which overshadows the increased protective effect at higher temperatures so evident in Tables 9 & 10.

It can therefore be concluded that much, if not all, of the protection offered by operating the presses at temperatures lower than 100° F. (as claimed in U.S. Pat. No. 4,681,769) as compared to temperatures above 100° F. is simply due to the higher residual oil levels (reduced extraction efficiency) and that, for any given residual oil content, and with rehydrated residual solids, the operating temperatures above 130° F. give superior results, not only in an increased extraction efficiency which allows for a continuous, high speed process with increased throughput rates and significantly reduced microbial activity, but most surprisingly in an increased color stability of both the extract and the residual press solids, particularly when the press solids are rehydrated.

#### Comparative Example

according to Bennett U.S. Pat. No. 4,681,769,—low temperature and pressure

As can be seen in Table 4, the press solids residual yield is much higher at temperatures below 100° F. and much higher (28.3% residual yield) than disclosed in U.S. Pat. No. 4,681,769 (10–15% residual yield). In Example 2, Table 4, the Model KEK 100 Screw Press used for the test was operated at about 100% of its rated capacity of 240 pounds per hour for typical oil seeds. In an effort to more closely model the residual yields of 10–15% (oil) in the cake as disclosed in U.S. Pat. No. 4,681,769, the feed rate for this test was set at about 95 pounds per hour, thus allowing more residence time in the press to expel more extract and to reduce the residual yield of the press residual solids to 10–15%.

The following Example according to Bennett is a two-step production run.

One lot, comprising about 300 lbs of 160 ASTA chili, ground to pass 20 mesh (USSS), was transferred to a ribbon blender and blended with 13.7% by starting weight of the ground chili of fortified soybean oil having 500 ASTA oleoresin for about 15 minutes and then allowed to stand for about 16 hours at room temperature (75° F.) before transfer to the feed hopper of an Egon Keller Model KEK-100 Screw Press. The feed hopper provides for a controlled flow of the mixture of chili and fortified oil to the press at a rate of about 95 lbs per hour of fresh ground chili, the equivalent of about 800 lbs per hour in a French Oil Mill Machinery Company F-44 press. Both these feed rates represent about 40% of the rated capacity of the respective screw presses on whole oil seeds. The production run was started with a cone setting of about 0.030 inches and with the internal worms configured so as to provide a pressure gradient of essentially little or no pressure up to about 500 pounds per square inch of pressure. At these low pressures and feed rates, effluent oil temperatures were maintained at less than 100° F. with cooling water as in Bennett, and the residual yield (oil) in the press residual solids averaged about 12.5%, just as prescribed by Bennett, who states that:

“Temperatures above 100° F. should be avoided inasmuch as higher temperatures cause oxidation with a resultant destruction of delicate flavor and/or color principle.”

With the press operating as described, the oil extracted, after centrifugation to remove the residual spice fines, assayed at about 1000 ASTA and the press cake residual solids fraction had a corresponding reduction in ASTA to about 115.

The press cake of the once-extracted ground fresh spice from the first pressing is further processed following the same procedure above described for the first blending/pressure extraction sequence using, however, fresh soybean oil as an additive in place of the fortified oleoresin soybean

oil. The fortified soybean oil extracted assayed at approximately 500 ASTA. This 500 ASTA fortified soybean oil extract is recycled as an extractant on fresh ground chili. The extracted chili powder cake from this extraction step had a corresponding reduction in ASTA value to an average of about 65 ASTA (ranged from 41 to 95 ASTA). The results of this low temperature, low pressure test are compared to results of high temperature, high pressure conditions in Example 2 and are shown in Table 12.

TABLE 12

Comparison of Low Temperature/Low Pressure and High Temperature/High Pressure

	Low Temp/Low Pressure, 16 hour batch mixing	High Temp/High Pressure Continuous from Example 2
Temperature, Deg F.	95	235
Pressure, psi	<500	20,000–30,000
Final Residual Solids ASTA	65	33
Final Residual Solids Yield	12.5%	9.3%
Final Residual Solids ASTA as a percent of Fresh Chili ASTA	41.5%	22%
ASTA loss in 1st mixing stage	7%	0%
ASTA loss in 2nd mixing stage	10.5%	0%
Final Oleoresin ASTA	1,000	1,000

It can clearly be seen, as is also shown in Example 2 (Effect of Varying Operating Temperatures), that the low temperature/low pressure batch process with extended contact times incurs significant color loss during the extended contact times necessary for low temperature/pressure extraction. In addition, the low temperature/pressure batch process does not remove the color as efficiently as with higher temperatures and pressures for any given size pressing operation.

The foregoing example can scientifically be scaled up or extrapolated into a comparative two-stage production run using two Model 44-F French Oil Mill Machinery Company presses, as follows:

One lot comprising about 3,840 lbs of 5% moisture, 160 ASTA, 20 mesh, fresh ground chili solids (*Capsicum annum*) is passed through a high speed, high shear, steam-jacketed paddle mixer on a continuous basis and fed directly into the press feed hopper at stage one, through the 1st stage press, into a 2nd stage paddle mixer, and then to the 2nd stage press. Soybean oil is added continuously through a metering pump into the paddle mixer at stage 2 at the rate of 13.7% by weight of the starting ground chili solids (525 lbs of oil for the 3840 lb run). The raw material chili solids are continuously fed at a rate of about 2,500 lbs per hour to the system with a total contact time in each paddle mixer of about 15 seconds. The temperature of the chili/oil mix exiting the paddle mixer is maintained at about 180° F. at stage 2 and about 150° F. at stage 1.

The oil/extract expelled from the second pressing stage is returned on a continuous basis to the paddle mixer at stage one wherein the oil/extract and fresh ground paprika are mixed in preparation for the first pressing stage. The oil/extract and fresh ground paprika exit the first stage paddle mixer and enter the first stage press at about 150° F., the temperature being controlled by the amount of steam on the paddle mixer jacket.

The concentrated oil/extract expelled from the first pressing stage is hydrated with water to about 75% by weight of



the fines (fine particulate solids) and gums and is then centrifuged and the hydrated fines and gums are added to the residual press solids from the final (second) pressing stage in a high shear mixer, after the solids have passed through a water-jacketed cooling screw.

The internal shaft and collar arrangements of the press are configured so as to provide internal pressures of about 20,000 to 30,000 PSI and cooling water is maintained at a flow rate through the bore of the shaft and through the cage cooling jackets so as to maintain an exit oil temperature at the external cage surface of about 180° to 200° F. and an exiting residual solid cake temperature of about 235° F. The residual cake solid is cooled in a water-jacketed screw conveyor to about 85° F. and water, in addition to the hydration water used to remove the fines and gums from the extract, is injected into the high shear, continuous mixer to rehydrate the cake to a water activity of about 0.6.

The concentrated extract exiting press stage one has an ASTA value of about 1,000 and the residual press solid cake exiting stage two has an ASTA value of about 45. The residual press solid cake has a reddish-brown appearance typical of lightly toasted chili powder. The aerobic plate count of the residual solid cake is about 70,000.

The same test is repeated (according to the Bennett Example). No heat is applied during the mixing stages and the press internals are reconfigured so as to provide for minimal friction and compression and the resultant minimal heat generation during the pressing operations. The working compression is supplied primarily by the cone at the cake discharge and is maintained at about 500 PSI. Oil is added at a rate of about 13.7% by weight of the starting paprika solids (525 lbs for the 3840 lb batch) and mixed in a ribbon blender for sixteen hours and then fed at ambient temperature (about 75° F.) to the pressing system. The feed rate through the pressing stages is maintained at 800 pounds per hour. Cooling water is supplied to the internal bore of the shafts and the cooling jackets to maintain exit oil temperatures of less than 100° F. on both the expelled oil and the residual press cake. The extract exiting the first pressing stage of the press is centrifuged without hydration of the gums and fine particulate solids.

The concentrated extract exiting press stage one has an ASTA value of about 1,000 and the residual cake solid has an ASTA value of about 65. The appearance of the cake is lacking the brownness characteristic of commercially-available ground paprika and chili powder and would require a separate browning step to make it acceptable for common uses. The material is difficult to regrind due to the high level of residual extractable yield left in the cake, it is not flowable, and it must be combined with other solid materials to make an acceptable product for sale. The aerobic plate count is about 220,000. A comparison of the results of the two tests is shown in Table 13.

TABLE 13

	High Temperature Pressing	Low Temperature Pressing
Mixing Time, Soy Oil	Continuous, (Seconds)	16 Hours
Fresh Oil Temp, °F.	75	75
Oil/Chili Stage 1 at Press Feed, °F.	150	75
Oil/Chili Stage 2 at Press Feed, °F.	180	75
Cake after Stage 1 Pressing, °F.	225	95
Cake after Stage 2 Pressing, °F.	235	95
ASTA Value, Extract	1000	995
½ Life, Extract, 65° C., Hours	25	16

TABLE 13-continued

	High Temperature Pressing	Low Temperature Pressing
ASTA Value, Press Cake	45	65
½ Life, Cake, 72° F., Weeks	32	10
Color Recovery, Extract	75%	51%
Aerobic Plate Count, Extract	70,000	2,000,000
Aerobic Plate Count, Cake	2,000	220,000
Overall Color Recovery (Extract and Cake)	98%	91%
Visual appearance, Cake	Red-brown	Red-tan
Throughput rate, lbs per hour	2,500	800

It is readily apparent that there are substantial advantages to higher temperatures and pressures. The color recovery is enhanced, there is a 50% increase in yield to the extract, the rate for a given press size is increased by over 300%, the color stability of the extract is improved by 65%, the color stability of the residual solids cake is improved by 300%, and the aerobic plate count is reduced by a factor of greater than 30 in both the extract and the residual cake; all without the oxidative color losses that are alleged to be an obstacle in U.S. Pat. No. 4,681,769.

It is thereby seen that an improved countercurrent process for the extraction of Capsicum solids using an edible solvent, whereby improved yields of both extract and residual solids are obtained, whereby both the extract and the residual solids have improved color stability and freedom from bacterial contamination due to the higher temperatures employed, whereby due to optional advantageous rehydration of residual solids and level of water activity employed an improved color stability in the residual solids is attained, whereby an extract in the form of a clear solution can be obtained by removal of gums and particulate solids in the form of their insoluble hydrates, whereby even greater color stability can be effected by the employment of edible antioxidants in the solvent utilized, and whereby controlled browning of the residual solids may be conveniently effected, all without the expected disadvantages of employing higher temperatures as clearly indicated by the prior art, and whereby all of the stated objects of the invention have been accomplished, has been provided.

It is to be understood that the invention is not to be limited to the exact details of operation, or to the exact compositions, methods, procedures, or embodiments shown and described, as obvious modifications and equivalents will be apparent to one skilled in the art, and the invention is therefore to be limited only by the full scope which can be legally accorded to the appended claims.

I claim:

1. A continuous multistage mixing, high pressure pressing, and countercurrent extraction process for the production of a concentrated edible extract and edible residual solids, both of reduced bacterial content, with the extract having enhanced resistance to oxidative deterioration of carotenoid pigments therein, and both of which contain carotenoid pigments, flavor, and aroma, from plant material solids of the genus *Capsicum*, comprising the following steps:

subjecting said Capsicum solids to a countercurrent extraction process involving a plurality of mixing and pressing stages, including first and last mixing stages and first and last pressing stages, together with five to about fifty percent by weight of an edible solvent, to produce an extract and residual solids, continuously returning the extract from each pressing stage to the previous mixing stage, and finally

separating the extract from the first pressing stage and separating the residual solids from the last pressing stage, all pressing stages being carried out at a temperature of at least 130° F.

2. A process of claim 1, wherein the temperature is 130° to about 450° F.

3. A process of claim 1, wherein the Capsicum solids are subjected to internal pressures in the press stages of at least 6,000 pounds per square inch.

4. A process of claim 1, wherein the weight of the edible solvent is 5% to about 20% by weight of the Capsicum solids.

5. A process of claim 1, wherein the moisture content of the starting Capsicum solids is less than 6% by weight, and wherein bacterial count reduction is effected at this low moisture content, thereby avoiding undesirable loss of volatile flavor and aroma constituents and avoiding the development of cooked, off flavors and aromas which occur at higher moisture contents.

6. A process of claim 1, wherein the Capsicum solids extracted in the process are selected from the group consisting of paprika, red pepper, and chili.

7. A process of claim 1, wherein the edible solvent is selected from the group consisting of soybean oil, corn oil, cottonseed oil, rapeseed oil, peanut oil, mono-, di-, or triglycerides, lecithin, edible essential oils, sesame oil, edible alcohols, hydrogenated or partially hydrogenated fats or oils, polyoxyethylene sorbitan esters, limonene, edible animal fats or oils, mixtures thereof, and edible derivatives thereof.

8. A process of claim 1, wherein fine particulate solids are filtered or centrifuged from the extract and alternatively discarded, returned to a mixing or pressing stage of the process, or incorporated in the final residual solids.

9. A process of claim 1, which includes the steps of hydrating the final extract to add water to the extent of 5% to 200% by weight of the gums and fine particulate solids therein and filtering or centrifuging to remove said gums and solids.

10. A process of claim 9 including the step of returning the separated hydrated gums and solids to the final residual solids.

11. A process of claim 1, including the step of rehydrating the final residual solids with water to a water activity greater than 0.3  $A_w$  for color stabilization thereof.

12. A process of claim 11, wherein the solids are rehydrated to a water activity of about 0.4 to 0.6  $A_w$ .

13. A process of claim 1, wherein an effective color-stabilizing amount of an edible antioxidant or chelator is included in the edible solvent.

14. A process of claim 13, wherein the antioxidant comprises an antioxidant selected from the group consisting of lecithin, ascorbic acid, citric acid, tocopherol, ethoxyquin, BHA, BHT, TBHQ, tea catechins, sesame, and the antioxidant activity from an herb of the Labiatae family.

15. A process of claim 14, wherein the antioxidant comprises a naturally-occurring antioxidant from an herb of the family Labiatae or powdered ascorbic acid.

16. A process of claim 15, wherein the antioxidant comprises the antioxidant activity from an herb selected from the group consisting of rosemary, thyme, and sage.

17. A process of claim 1, wherein the temperature is greater than 180° F.

18. A process of claim 1, wherein the temperature is between about 180° F. and 235° F.

\* \* \* \* \*

# **EXHIBIT G**

**United States Patent** [19]**Tsujiwaki et al.**[11] **Patent Number:** **5,840,945**[45] **Date of Patent:** **Nov. 24, 1998**

[54] **METHOD FOR REFINING AND MANUFACTURING FATS AND OILS CONTAINING POLYUNSATURATED FATTY ACIDS**

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[30] **Foreign Application Priority Data**

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[51] **Int. Cl.<sup>6</sup>** ..... **C07C 51/43**

[52] **U.S. Cl.** ..... **554/192; 554/175; 554/191; 554/193; 554/196**

[58] **Field of Search** ..... **554/191, 196, 554/192, 175, 193**

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[57]

**ABSTRACT**

A method of refining and manufacturing a fat and oil containing polyunsaturated fatty acids which prevents production of peculiar fishy smells when such a fat and oil is added to food materials and while it is stored by preventing oxidation of PUFA's and restraining conversion of odor-emanating precursors into odor-emanating substances. A fat and oil, such as a marine animal oil, containing polyunsaturated fatty acids with 18 or more carbon atoms and three or more double bonds such as EPA and DHA is alkali refined and bleached. The thus alkali refined and bleached fat and oil is refined by bringing 100 parts by weight of it into contact for 10 minutes or more at 5°–80° C. with not less than 0.1 part by weight of powdered or granulated diatomaceous earth formed by calcining with a flux added such as sodium carbonate or sodium chloride. The fat and oil thus obtained is filtered and deodorized by steam distillation under vacuum.

**3 Claims, No Drawings**

# METHOD FOR REFINING AND MANUFACTURING FATS AND OILS CONTAINING POLYUNSATURATED FATTY ACIDS

## BACKGROUND OF THE INVENTION

This invention relates to a method for refining and manufacturing fats and oils containing polyunsaturated fatty acids and used as foods, drugs and cosmetics.

Docosahexaenoic acid (hereinafter abbreviated to DHA), eicosapentaenoic acid (abbreviated to EPA) and  $\alpha$ -linolenic acid are polyunsaturated fatty acids (abbreviated to PUFA) having 18 or more carbon atoms and three or more double bonds. Such PUFA's lower cholesterol and neutral fat levels in the blood, and suppress aggregation of platelets. Among such PUFA's, DHA, which is present especially in rich amounts in the brain, retinas, testicles and human milk, is believed to be a substance essential for the development of the nervous system.

Therefore, efforts have been made to develop foods and medicines that contain marine animal fats and oils, especially fish oils, which contain PUFA in large amounts.

But PUFA-containing fats and oils have peculiar unpleasant smells. For example, fish oils have fishy smells. Thus, such oils are not used so widely in food materials.

Fish oils obtained from tunas and skipjack, which contain DHA in high concentrations, can be made tasteless and odorless by refining and deodorization. But while the thus refined and deodorized fish oils are stored, the PUFA's double bonds are oxidized by oxygen in the air. Mainly due to this, the oils begin to emanate peculiar fishy smells again.

In order to suppress PUFA's oxidation, antioxidants such as tocopherols, ascorbic acid or lecithin are often added. But it is still difficult to prevent the recurrence of fishy and other unpleasant smells.

In order to hide such recurring smells, odor suppressors and masking agents were used. But it was still impossible to sufficiently suppress odors. Also, the effects of such agents are short-lived, so that they cannot offer a fundamental solution to the odor problem.

From the fact that the addition of antioxidants cannot prevent perfectly the recurrence of odors, the inventors of the present invention, thought that factors other than oxidation may be playing a role in the recurrence of odors. More specifically, they thought that trace amounts of precursors of the odor-emanating substances that remain unremoved by the conventional refining method might convert into odor-emanating substances which, in cooperation with the odors due to oxidation of the double bonds in PUFA's, produce peculiar fishy odors, lowering the market value of the oil products.

Similarly, PUFA containing vegetable oils such as perilla oil and linseed oil also produce unpleasant odors in the same mechanism as with fish oils.

An object of this invention is to provide an improved method of refining and manufacturing a fat and oil containing polyunsaturated fatty acids which prevents production of peculiar fishy smells when such a fat and oil is added to food materials and while it is stored by preventing oxidation of PUFA's and restraining conversion of odor-emanating precursors into odor-emanating substances.

## SUMMARY OF THE INVENTION

According to this invention, there is provided a method of refining an oil containing polyunsaturated fatty acids having

18 or more carbon atoms and three or more double bonds, the method comprising the step of bringing 100 parts by weight of the oil into contact for 10 minutes or more at 5°-80° C. with not less than 0.1 part by weight of powdered diatomaceous earth formed by calcining with a flux added.

There is also provided a method of manufacturing an oil containing polyunsaturated fatty acids, the method comprising the steps of alkali refining and bleaching an oil containing polyunsaturated fatty acids having 18 or more carbon atoms and three or more double bonds, bringing 100 parts by weight of the oil into contact with 0.1 part by weight of powdered diatomaceous earth formed by calcining with a flux added for 10 minutes or more at 5°-80° C., filtering the oil, and deodorizing the oil by steam distillation under vacuum.

The PUFA-containing oil used in this invention may be any oil containing polyunsaturated fatty acids having 18 or more carbon atoms and three or more double bonds. Preferably, the number of carbon atoms is 18-22 and the number of double bonds is 3-6. Such an oil should contain DHA, EPA or  $\alpha$ -linolenic acid. Such a PUFA-containing oil may be at least one fish oil selected from the group consisting of skipjack oil, tuna oil, sardine oil, Alaska pollack oil, salmon oil, squid oil, mackerel pike oil, horse mackerel oil and mackerel oil, a marine animal oil such as whale oil, or a vegetable oil such as perilla oil and linseed oil, provided it contains 5% by weight or more of PUFA. Also, the oil used in the invention may be a marine animal oil, a vegetable oil, or any combination of a marine animal oil, a vegetable oil, a non-marine animal fat, and a different vegetable oil containing less than 5% by weight of PUFA.

We will discuss the diatomaceous earth used in this invention.

Generally known diatomaceous earth is fossilized siliceous shells of diatoms or aquatic single-cell phytoplanktons with their cell contents lost. Such porous diatomaceous earth is refined and used as industrial materials such as a filtering assistant, filler and building material. Large-scale deposits of such diatomaceous earth in Japan include an oceanic deposit in Akita prefecture and freshwater deposits in Oita and Okayama prefectures.

Commercially available diatomaceous earth is produced by refining raw ores of diatomaceous earth and comes in the following forms: uncalcined powder formed by pulverizing and optionally adjusting its composition and particle diameter; calcined powder formed by calcining such uncalcined powder at 900°-1200° C., powder calcined after adding 4-8% by weight of a flux such as sodium carbonate or sodium chloride (flux-calcined powder), molded articles molded into the shape of a cylinder, ring, sphere, plate or box by optionally adding forming additives such as binders; and amorphous granules obtained by crushing the molded articles.

Such diatomaceous earth originating from diatom shells has numerous pores about 100-1000 nm in diameter. Due to complicated shapes of the shells with no cell contents, the surface of such diatomaceous earth is undulated in an extremely complicated manner, creating a high porosity of about 60-90%.

The diatomaceous earth used in this invention is a powder obtained by calcining after adding 0.5-12% by weight of at least one flux selected from the group consisting of alkali metallic salts, alkali earth metallic salts, hydroxides of alkali metals and hydroxides of alkali earth metals to the above-mentioned uncalcined powder of diatomaceous earth.

The flux used in calcining the diatomaceous earth is added to the earth before calcining and used to aggregate the shells into large masses by partially melting them during sintering.

The flux used in the invention may be an alkali metallic salt such as sodium carbonate or sodium chloride, an alkali earth metallic salt such as calcium carbonate or magnesium carbonate, a hydroxide of an alkali metal or alkali earth metal such as sodium hydroxide, or a mixture thereof.

The content of flux is preferably 0.5–12% by weight, though it depends on the kind of diatomaceous earth used. Less than 0.5% by weight of flux cannot form sufficiently large aggregates of shells, making it impossible to sufficiently improve various properties of the PUFA-containing oil (such as flavor and resistance to oxidation). If more than 12% by weight, it will melt the diatomaceous earth excessively during calcining, forming oversized, difficult-to-handle aggregates.

The PUFA-containing oil is refined by bringing it into contact with powdered diatomaceous earth formed by calcining with a flux added, after subjecting the oil to alkali refining (separation of free fatty acids with alkalis) and bleaching (adsorption e.g. using activated clay or activated carbon at high temperatures).

The refining step may be carried out in a batch process or a continuous process in which a column is used.

In the batch process, the flux-calcined diatomaceous earth is added by 0.1 part by weight or more, practically 0.1–10 parts by weight, per 100 parts by weight of PUFA-containing oil. After mixing them, the mixture is filtered to batch off the oil. If the amount of diatomaceous earth added is less than 0.1 part by weight, it will be impossible to sufficiently improve the flavor of the PUFA-containing oil and its resistance to oxidation after refinement. Adding more than 10 parts by weight is meaningless because no further improvement in such properties can be expected, though no harm is done, either. Preferably, the flux-calcined diatomaceous earth should be added by 3–8 parts by weight per 100 parts by weight of PUFA-containing oil.

The PUFA-containing oil should be brought into contact with the flux-calcined diatomaceous earth at an oil temperature from 5° to 80° C. If this temperature is lower than 5° C., the PUFA-containing oil will half solidify, making the handling difficult. At temperatures higher than 80° C., it is impossible to improve the flavor and the resistance to oxidation of the refined PUFA-containing oil as expected. Rather, the effects will be lower at such high temperatures. Preferable contact temperature is 25°–45° C.

The PUFA-containing oil should be brought into contact with the flux-calcined diatomaceous earth for 10 minutes or longer to achieve the effects.

In the continuous process in which a column is used, 100 parts by weight of PUFA-containing oil is passed through the column filled with 50–100 parts by weight of flux-calcined diatomaceous earth. The contact temperature is determined on the same principle as in the batch process. The PUFA-containing oil should be retained in the column for 10 minutes or longer to achieve the object of the invention.

It is possible to further improve the oxidation resistance and the flavor of the PUFA-containing oil by deodorizing it by conventional steam distillation under vacuum after refining.

#### (EXAMPLES)

The types and physical properties of the diatomaceous earths used in Examples and Comparative Examples are shown in Table 1.

It was impossible to form diatomaceous earth (C) in Table 1, which contained 15% by weight of flux, into powder form.

#### (Example 1)

1000 grams of skipjack oil (containing 25% DHA and 6% EPA) as a PUFA-containing oil subjected to alkali refining and bleaching that are ordinarily carried out in refining food oils was put in a 2-liter beaker, and agitated for one hour at 40° C. for contact treatment after adding 50 grams of diatomaceous earth (A) in Table 1. After this contact treatment, the oil was separated from the diatomaceous earth by filtering and deodorized at 215° C. for one hour by steam distillation in which steam is blown in under a reduced pressure of 3 Torr.

The DHA content in the fatty acid in the deodorized oil was measured by gas chromatography. The DHA content was 23.5%, while the EPA content was 5.8%.

In order to determine the odor level and peroxide level of the oil after storing, 100 grams of the deodorized oil was put in a 200-milliliter ground-glass bottle after adding 3000 ppm of mixed tocopherols (made by EIZAI). After storing it for 30 hours at 60° C., it was subjected to an odor sensory test and peroxide measurement test.

In the odor sensory test, three each male and female adults were selected as panellists to evaluate the odor level in points graduated to the first decimal place by the following standards. The points shown in Table 2 are the average of the points given by the panellists. Table 2 also shows the peroxide content (meq/kg).

5.0: no fishy smells at all

4.0: slight fishy smells

3.0: moderate fishy smells

2.0: strong fishy smells

1.0: stimulative fishy smells

#### (Examples 2–8)

The same oil used in Example 1 was brought into contact with diatomaceous earth in exactly the same way as in Example 1, except that the diatomaceous earth (A) was replaced with diatomaceous earth (B) (Example 2), flux-calcined diatomaceous earth (D) (Example 3), flux-calcined diatomaceous earth (E) (Example 4), flux-calcined diatomaceous earth (F) (Example 5), flux-calcined diatomaceous earth (G) (Example 6), flux-calcined diatomaceous earth (H) (Example 7), and flux-calcined diatomaceous earth (I) (Example 8). Then, they were subjected to filtering, deodorization and addition of tocopherols in the same manner as in Example 1. After measuring their DHA contents, they were stored under exactly the same conditions as in Example 1 and their odor levels and peroxide levels were determined. The results are shown in Table 2.

#### (Comparative Examples 1–5)

The same oil used in Example 1 was brought into contact with diatomaceous earth in exactly the same way as in Example 1, except that the diatomaceous earth (A) was not used (Comparative Example 1), powdered activated carbon was used (Comparative Example 2), silica gel was used (Comparative Example 3), uncalcined diatomaceous earth (J) containing no flux was used (Comparative Example 4), and calcined diatomaceous earth (K) containing no flux was used (Comparative Example 5). They were subjected to filtering, deodorization and addition of tocopherols in the same manner as Example 1. After measuring their DHA contents, they were stored under exactly the same conditions as in Example 1 and their odor levels and peroxide levels were determined. The results are shown in Table 2.

## 5

As shown in Table 2, for Comparative Example 2, which used activated carbon, and Comparative Example 5, which used calcined diatomaceous earth (K) without a flux, the fish oil smelled fishy when it had been stored for 30 hours, though its peroxide level was low. For Comparative Example 3, which used silica gel, and Comparative Example 4, in which was used uncalcined dried diatomaceous earth (J), the fish oil smelled fishy and the peroxide level was high after 30 hours' storage.

In contrast, Examples 1-8, which were treated by bringing them into contact with diatomaceous earth calcined with a flux added, were markedly low in peroxide level compared with Comparative Examples 1-5 and achieved good results in the sensory test.

## (Examples 9-11)

The same oil used in Example 1 was brought into contact with diatomaceous earth in exactly the same way as in Example 1, except that the contact treatment was carried out for one hour at 25° C. using 30 grams of diatomaceous earth (A) shown in Table 1 (Example 9), that the contact treatment was carried out for 10 minutes at 5° C. using 1 gram of diatomaceous earth (A) (Example 10), and that the contact treatment was carried out for 10 minutes at 80° C. using 1 gram of diatomaceous earth (A) (Example 11). The oils obtained were then filtered and deodorized, and their DHA contents were measured in the same way as in Example 1. After measuring their DHA contents, they were stored under exactly the same conditions as in Example 1 and their odor levels and peroxide levels were determined. The results are shown in Table 3.

## (Comparative Examples 6)

The same oil used in Example 1 was brought into contact with diatomaceous earth, filtered and deodorized in exactly the same way as in Example 1, except that the contact treatment was carried out for one hour at 100° C. using diatomaceous earth (A) shown in Table 1. The oils thus obtained were stored under exactly the same conditions as in Example 1 and their odor levels and peroxide levels were determined. The results are shown in Table 3.

As shown in Table 3, Examples 9-11, which satisfy all the treatment conditions with diatomaceous earth, were low in peroxide level and had little fishy smells after storage. But when the oil was treated at 100° C. by bringing it into contact with diatomaceous earth, the oil produced fishy smells though its peroxide level was kept low, in spite of the fact that the diatomaceous earth used had been treated with a flux added.

## (Example 12)

30 grams of diatomaceous earth (A) shown in Table 1 was filled into a column 105 cm<sup>2</sup> in volume, and the column was placed in a constant temperature bath kept at a constant temperature of 40° C. Skipjack oil pretreated (alkali refined and bleached) under the same conditions as in Example 1 was passed through the column at a rate of 1 milliliter/minute for an hour.

The oil obtained was stored under the same conditions as in Example 1 and its peroxide level and odors were measured. The results are shown in Table 4.

As shown in Table 4, when 100 parts by weight of diatomaceous earth was brought into contact with 100 parts by weight of PUFA-containing oil, the peroxide level and fishy smells after storage were the lowest.

## 6

## (Example 13)

A mixture of 750 grams of skipjack oil and 250 grams of corn oil (DHA 16.7%, EPA 3.5%) as a PUFA-containing oil was treated in exactly the same way as in Example 9. The oil thus obtained was stored under the same conditions as in Example 9 and its peroxide level and odors were measured. The results are shown in Table 5.

## (Comparative Example 7)

The same oil used in Example 13 was treated in exactly the same manner as in Example 13 except that it was not treated with diatomaceous earth calcined with a flux added. The oil obtained was stored under the same conditions as in Example 13 and its peroxide level and odors were measured. The results are shown in Table 5.

As shown in Table 5, it is impossible to suppress the peroxide level and fishy smells of a PUFA-containing oil simply by adding corn oil thereto. But by treating such a mixture of oils with diatomaceous earth (A) calcined with a flux, its peroxide level and fishy smells after storage decreased markedly.

## (Example 14)

500 grams of alkali refined and bleached perilla oil (containing 19.5% oleic acid, 15.9% linoleic acid and 59.2%  $\alpha$ -linolenic acid) as a PUFA-containing oil was put in a 1-liter beaker, together with 25 grams of diatomaceous earth (A) shown in Table 1, and agitated for one hour at 40° C. for contact treatment. After the treatment, the diatomaceous earth was separated by filtering. The oil thus obtained was deodorized by steam distillation for one hour at 215° C. under a reduced pressure of 3 Torr.

The PUFA content in the fatty acid in the deodorized oil was measured by gas chromatography. The content of  $\alpha$ -linolenic acid was 57.9%.

This oil was stored under the same conditions as in Example 1, and 48 hours later, its peroxide level and odor level were measured. The results are shown in Table 6. In the odor sensory test, the odor level was evaluated in the following four stages, because unlike fish oils, this oil has no strong smells.

- ⊙ no peculiar smells at all
- no smells
- Δ slight smells
- X intense smells

## (Comparative Example 8)

The same oil used in Example 14 were treated in exactly the same way as in Example 14 except that no diatomaceous earth was used. The oil obtained were stored under the same conditions as in Example 14 and its peroxide level and odor level were measured. The results are shown in Table 6.

As shown in Table 6, it is possible to suppress the peroxide level and odor levels of even perilla oil, a PUFA-containing vegetable oil by treating it with diatomaceous earth calcined with a flux added.

According to this invention, an oil containing polyunsaturated fatty acids is refined by bringing it into contact with diatomaceous earth calcined with a flux added. The refining and manufacturing method can suppress fishy or other peculiar odors which are produced due to oxidation of PUFA and conversion of odor-emanating precursors into odor-emanating substances when the fat and oil is added to a food or while a food containing such a fat and oil is stored.

TABLE 1

Type of diatomaceous earth	Type of flux used	Item		
		Content of flux in wt %	Average particle size in $\mu\text{m}$	Condition of diatomaceous earth
Flux-calcined diatomaceous earth	(A) Sodium carbonate	7	33	White powder
Flux-calcined diatomaceous earth	(B) Sodium carbonate	0.5	18	Pink powder
Flux-calcined diatomaceous earth	(C) Sodium carbonate	15	—	Not obtained
Flux-calcined diatomaceous earth	(D) Sodium carbonate	7	500	White granule
Flux-calcined diatomaceous earth	(E) Sodium carbonate 50% Sodium chloride 50%	6	33	White powder
Flux-calcined diatomaceous earth	(F) Potassium chloride	6	33	White powder
Flux-calcined diatomaceous earth	(G) Calcium carbonate	6	33	Salmon-pink powder
Flux-calcined diatomaceous earth	(H) Magnesium carbonate	6	33	Salmon-pink powder
Flux-calcined diatomaceous earth	(I) Sodium hydroxide	5	33	White powder
Dried diatomaceous earth	(J) —	—	11	Light brown powder
Calcined diatomaceous earth	(K) —	—	13	Salmon-pink powder

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TABLE 2

No.	Item	
	Sensory evaluation point	Peroxide number in meq/kg
Example 1	4.1	2.70
Example 2	3.9	3.45
Example 3	4.2	2.50
Example 4	4.0	2.89
Example 5	3.9	3.11
Example 6	4.0	3.05
Example 7	3.8	2.96
Example 8	3.8	4.09
Comparative example 1	2.5	9.22
Comparative example 2	2.5	5.91
Comparative example 3	2.5	5.30
Comparative example 4	3.3	5.81
Comparative example 5	3.5	5.15

TABLE 3

No.	Item	
	Sensory evaluation point	Peroxide number in meq/kg
Example 9	4.0	2.34
Example 10	3.8	3.85
Example 11	3.8	4.17
Comparative example 6	3.3	4.77

TABLE 4

No.	Item	
	Sensory evaluation point	Peroxide number in meq/kg
Example 12	4.2	2.47

TABLE 5

25

No.	Item	
	Sensory evaluation point	Peroxide number in meq/kg
Example 13	4.2	1.89
Comparative example 7	2.8	8.26

30

TABLE 6

35

No.	Item	
	Sensory evaluation point	Peroxide number in meq/kg
Example 14	⊙	1.43
Comparative example 8	Δ	1.69

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What is claimed is:

1. A method of refining at least one member selected from the group consisting of a fat and an oil containing polyunsaturated fatty acids having 18 or more carbon atoms and three or more double bonds, said method comprising the step of bringing 100 parts by weight of said at least one member into contact for 10 minutes or more at 5°–80° C. with not less than 0.1 part by weight of powdered or granulated diatomaceous earth formed by calcining with a flux added.

2. A method as claimed in claim 1 wherein said flux is selected from the group consisting of alkali metallic salts, alkali earth metallic salts, hydroxides of alkali metals and hydroxides of alkali earth metals and is added in an amount of 0.5–12% by weight to said powdered or granulated diatomaceous earth.

3. A method as claimed in claim 1 or 2 wherein said at least one member to be refined contains 5% by weight or more of polyunsaturated fatty acids and is a marine animal oil, a vegetable oil, or any combination of a marine animal oil, a vegetable oil, a non-marine animal fat, and a different vegetable oil containing less than 5% by weight of polyunsaturated fatty acid.

\* \* \* \* \*



# **EXHIBIT H**

# United States Patent [19]

Kyle et al.

[11] Patent Number: **5,407,957**

[45] Date of Patent: **Apr. 18, 1995**

- [54] **PRODUCTION OF DOCOSAHEXAENOIC ACID BY DINOFLAGELLATES**
- [75] Inventors: **David J. Kyle**, Catonsville; **Sue E. Reeb**; **Valerie J. Sicotte**, both of Baltimore, all of Md.
- [73] Assignee: **Martek Corporation**, Columbia, Md.
- [21] Appl. No.: **479,135**
- [22] Filed: **Feb. 13, 1990**
- [51] Int. Cl.<sup>6</sup> ..... **A23D 9/00; C12P 7/64; C07C 69/52; A61K 31/225**
- [52] U.S. Cl. .... **514/547; 426/33; 426/601; 560/205; 435/134; 514/549; 514/552**
- [58] Field of Search ..... **435/134, 136; 560/205, 560/190, 191; 426/33, 601; 514/549, 552, 547**

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## [57] ABSTRACT

This invention relates to a process for producing a single cell edible oil containing DHA, to the oil itself and to uses for the oil. Marine microorganisms are cultivated in fermentors and induced to produce the single cell oil which subsequently is recovered by extraction with solvents.

25 Claims, 1 Drawing Sheet

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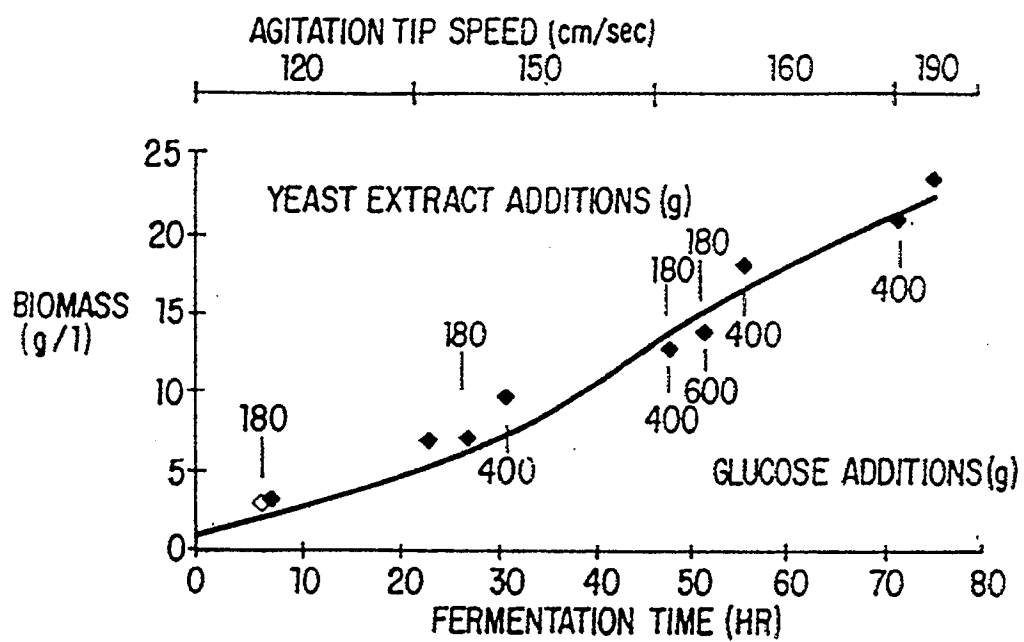


FIG. 1

## PRODUCTION OF DOCOSAHEXAENOIC ACID BY DINOFLAGELLATES

### BACKGROUND OF THE INVENTION

This invention relates to edible, single-cell oil containing docosahexaenoic acid (DHA). The invention also relates to methods of producing such oil containing DIN in commercially viable yields and to products containing the oil.

DHA is an omega-3-fatty acid and is the most abundant long chain polyunsaturated fatty acid (PUFA) in the grey matter of the brain. Omega-3-fatty acids in general are known to be beneficial in reducing the incidence of coronary heart disease [Lands, *Fish and Human Health* (1986) Academic Press]. However, the metabolism of omega-3-fatty acids is not well understood. Thus, precise clinical dosages and efficacy remain unknown.

Cold water marine fish are a known source of omega-3-fatty acids, including DHA. U.S. Pat. No. 4,670,285 discloses the use of fish oil from fish such as menhaden and herring as a source of C<sub>22</sub> omega-3-fatty acids. Indeed, fish oils are the primary commercial source of omega-3-fatty acids. Often, however, fish oils are unusable for human consumption because of contamination with environmental pollutants such as PCB's.

There also are problems associated with the recovery of fish oils containing DHA. Such oils often have a fishy odor and unpleasant tastes associated with the acids. These tastes render the oils unsatisfactory for use in edible compositions such as baby food and infant formulas.

Marine microorganisms also are known to contain DHA. In particular, various species of dinoflagellates are known to contain DHA. Harrington et al., "The Polyunsaturated Fatty Acids of Marine Dinoflagellates" *J. Protozoal*, 17:213-219 (1970), characterize the fatty acid content of eight photosynthetic and one heterotrophic marine dinoflagellates, and conclude that the dinoflagellates are a primary producer group of docosahexaenoic acid and contribute substantial amounts of that compound to the marine food chain.

Successful cultivation of dinoflagellates to produce an edible oil containing DHA has not been achieved. Dinoflagellates in general are very slow growing and are shear sensitive. Guillard et al., *Dinoflagellates*, (1984) Academic Press. The prior art discloses that even a small amount of agitation in the culturing vessel reduces growth of the cultures. However, such agitation would be necessary to achieve adequate oxygenation in order to maximize growth for commercial production.

DHA is thought to be essential for the proper brain development of infants because, as noted above, it is the most abundant long chain PUFA in the brain. Although a metabolic pathway exists in mammals for the biosynthesis of DHA, this pathway is bioenergetically unfavorable [Crawford, *P. AOCS. Short Course in Polyunsaturated Fatty Acids and Eicosanoids*, pp. 270-295 (1987)] and mammals, like fish, are thought to obtain most of their DHA from dietary sources. In the case of infants, the most likely source would be human milk. Indeed, DHA is the most abundant PUFA in human milk. Generally, however, DHA is absent from infant formulas. U.S. Pat. No. 4,670,285 does disclose an infant formula containing omega-3-fatty acids. However, the acids utilized therein are obtained from fish oil and have asso-

ciated therewith the unpleasant characteristics previously described. Furthermore, fish oils generally contain another omega-3-fatty acid, eicosapentaenoic acid (EPA), an undesirable component in infant formulas because of its prolonged anticoagulant effects.

Accordingly, it is an object of the present invention to provide a single-cell edible oil containing DHA. Preferably this oil will have no significant quantities of other polyunsaturated fatty acids (PUFA's), i.e. greater than about 2% of the total fatty acid content. In general, it is an object of the present invention to produce single-cell oil in commercially viable yields. The oil, characterized herein as a "designer" oil, after extraction can be used in infant formulas, baby foods, dietary supplements and pharmaceuticals.

In addition, it would be desirable to acquire further knowledge of the metabolic pathway of omega-3 fatty acids. Isotopically labeled DHA would be of great utility in this regard. However, to date, no method has been known to produce abundant quantities of isotopically labelled DHA. Thus, it also is an object of the present invention to provide isotopically labeled DHA in sufficient quantities to undertake such research.

### SUMMARY OF THE INVENTION

The present invention relates to the cultivation of microorganisms, notably dinoflagellates, in a fermentor, induction of those microorganisms to produce significant quantities of single cell oil containing a high proportion of DHA and recovery of that oil. As used herein, "single cell oil" refers to a triglyceride product of a unicellular organism. The present invention also includes mutant organisms capable of producing enhanced quantities of single-cell oil containing at least about 20% by weight DHA and includes single cell oil containing DHA.

The present invention provides an economical method of obtaining enhanced levels of edible oils containing DHA. Additionally, the method permits the commercial cultivation of dinoflagellates in elevated cell densities.

Edible oils produced by the method of this invention lack unpleasant tastes and fishy odors and also are free of environmental contaminants often found in DHA-containing oils from conventional sources. Accordingly, the present invention further includes food products containing the oil of this invention.

### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graphic illustration of *C. cohnii* biomass accumulation over time with the addition of various nutrients.

### DETAILED DESCRIPTION OF THE BEST MODE OF PRACTICING THE INVENTION

In accordance with the present invention, microorganisms capable of producing a single cell oil containing DHA are cultivated in a fermentor in a nutrient solution capable of supporting the growth of such organisms. Preferably the single cell oil will contain at least about 20% by weight DHA.

Any microorganisms capable of producing a single-cell edible oil containing DHA can be used in the present invention. For example, photosynthetic diatoms can be used. Preferred microorganisms are marine dinoflagellates, including *Cryptocodinium sp.* Especially preferred is *Cryptocodinium cohnii*, an obligate hetero-

troph requiring a reduced carbon source for growth. *C. cohnii* preferred because it contains a fatty acid profile in which DHA is the only PUFA present in sufficient quantities (greater than about 1% of the total amount of PUFAs). Samples of this organism, designated MK8840, have been deposited with the American Type Culture Collection at Rockville, Maryland, and assigned accession number 40750. As used herein, microorganism, or any specific type of microorganism, includes wild strains, mutants or recombinant types. Any microorganism which produces enhanced levels of oil containing DHA is considered to be within the scope of this invention. One of the features of the present invention is its recognition of the edible oil-producing capability of microorganisms such as dinoflagellates and the attendant solution to the problem of maintaining a reliable, economic source of such oils. Accordingly, wild-type and recombinant microorganisms designed to produce single cell oil containing DHA are an aspect of this invention. Such recombinant organisms would include those designed to produce greater quantities of DHA in the single cell oil, greater quantities of total oil, or both, as compared to the quantities produced by the same wild type microorganism, when provided with the same substrates. Also included would be microorganisms designed to efficiently use more cost-effective substrates while producing the same amount of single cell oil containing DHA as the comparable wild-type microorganism.

In general, those of skill in the art would not consider *C. cohnii* a suitable organism for cultivation in a fermentor. Previous workers have commented on the extremely complex mixture of nutrients required to successfully cultivate *C. cohnii*. Gold et al. *Protozoal*, 13:255-257 (1966); Guillard, et al. in "Dinoflagellates", Academic Press (1984); Henderson, et al., *Phytochemistry* 27:1679-1683 (1988). In contrast, the present invention achieves the cultivation of DHA-producing microorganisms in a simple medium containing glucose and yeast extract. Use of these components in a solution such as seawater provides economically significant growth rates and cell densities. For example, during the course of a 3-5 day fermentation, *C. cohnii* cell densities of at least 10 grams of biomass per liter of solution, and typically from 20 to about 40 grams per liter, can be attained. Such densities have not heretofore been attainable.

Although cultivation can occur in any suitable fermentor, preferably the organism is grown either in a stirred tank fermentor (STF) or in an air lift fermentor (ALF), both types known to those of skill in the art. When a STF is selected, agitation is provided using either Rushton-type high efficiency turbines or pitched-blade or marine impellers. Agitation and duration renews the supply of oxygen to the microorganisms. The rate of agitation normally is increased as the biomass increases, due to the increased demand for oxygen. It is desirable to keep the tip speed at not greater than about 500 cm/sec. Selection of strains of microorganisms which are capable of withstanding greater tip speeds without undergoing shear is within the purview of those of skill in the art. The use of such strains is expressly included in this invention.

As noted above, seawater is an acceptable medium for the nutrient solution. The seawater can be either natural, filtered or an artificial mix, each of which can be diluted to  $\frac{1}{2}$  strength with tap water or concentrated to 2 times normal strength. A preferred example is In-

stant Ocean ® (IO) brand artificial seawater. Although *C. cohnii* is a marine microorganism, some growth has been observed in zero salinity. The use of variants which grow well in reduced salinities is specifically encompassed by this invention. Micronutrients can be added and may be required. However, such micronutrients are known to those of skill in the art and generally are present in seawater or tap water. If the organism selected is heterotrophic, such as *C. cohnii*, then a carbon source is added.

Preferably, after addition of the seawater medium to the fermentor, the fermentor containing the medium is sterilized and cooled prior to adding the nutrients and a seeding population of microorganism. (Although it is acceptable to sterilize the nutrients together with the seawater, sterilization in this manner can result in a loss of available glucose.) The nutrients and microorganism can be added simultaneously or sequentially.

An effective seed concentration can be determined by those of skill in the art. When a STF is used, the addition of a population of from about 0.05 to 1.0 grams of dry weight equivalent per liter at the beginning of the fermentation is preferred. This is about  $10^5$  cells per ml. Thus, for a 30 liter fermentor, 1.5 liters of seeding media, containing viable cells at a density of 20 g dry weight per liter would be added.

Oxygen levels preferably are maintained at a D.O. of at least about 10% of air saturation level. Biosynthesis of DHA requires oxygen and, accordingly, higher yields of DHA require D.O. levels at from about 10% to 50% of air saturation levels. Agitation tip speeds of 150-200 cm/sec in combination with an aeration rate of 1VVM (volume of air/volume of fermentor per minute) provides D.O. levels of from about 20% to about 30% at biomass densities of about 25 g dry weight/liter of culture. Higher cell densities may require higher D.O. levels, which can be attained by increased aeration rates by  $O_2$  sparging, or by increasing the air pressure in the fermentor.

Acceptable carbon sources are known to those of skill in the art. For example, carbon can be provided to *C. cohnii* in the form of glucose. Other heterotrophs can use other reduced carbon sources, a matter easily determined by those of skill in the art, and autotrophs utilize carbon dioxide. *C. cohnii* will also grow on other reduced, more complex, carbon sources. Typically, a fermentation is initiated with about 10-20 g/liter glucose. More glucose is added during the fermentation as required. Alternatively, from about 80 to 150 g glucose/liter initially can be added, thereby minimizing the frequency of future additions. If glucose levels drop to zero, the culture can die within a few hours. The amount of carbon source provided to other organisms can readily be determined by those of skill in the art.

In addition to a reduced carbon source, a nitrogen source, such as yeast extract (YE), is provided to the medium. Commercially available yeast extract is acceptable. For example, DIFCO brand yeast extract can be used. The yeast extract is an organic nitrogen source also containing micronutrients. Other organic nitrogen sources can easily be determined by those of skill in the art. However, such compounds are more expensive than yeast extract. The use of variants capable of growing on urea or nitrates is within the scope of this invention. Typically, the fermentation is initiated with about 4-8 g YE/liter. More YE can be added as required. A typical fermentation run requires from about 25 to 50 g YE/liter over the course of the run. Accordingly, that

amount of YE can be added initially with a reduced need for further additions. The precise amount can be determined by those of skill in the art.

The cultivation can be carried out at any life-sustaining temperature. Generally *C. cohnii* will grow at temperatures ranging from about 15° C. to 34° C. Preferably the temperature is maintained at about 20°–28° C. Strains which grow at higher temperatures are preferred, because they will have a faster doubling time, thereby reducing the fermentation time. Appropriate temperature ranges for other microorganisms are readily determined by those of skill in the art.

The cultivation can be carried out over a broad pH range, typically from about pH 5.0 to 9.0. Preferably, a pH range of from about 7.0 to about 7.8 is used. The initial growth tends to acidify the medium. Addition of a base, such as KOH or NaOH, corrects this acidification. During the later stages of the fermentation, the culture medium tends to become alkaline. The addition of YE ordinarily is sufficient to maintain the pH in the desired range. However, if desired, inorganic acid pH controls can be used to correct alkalinity.

Production of the single cell oil is induced in the dinoflagellates by the imposition of a nitrogen deficiency. Such deficiencies are caused by providing YE in a limiting amount such that the medium runs out of YE while available glucose remains. The present invention recognizes that it is the carbon source to nitrogen source ratio which promotes the efficient production of the single cell oil. Using glucose and YE as exemplary, a preferred ratio of carbon source to nitrogen source is about 2–4 parts glucose to 1 part YE. Similar ratios for other carbon and nitrogen sources can be calculated by those of skill in the art.

After induction of oil production, the culture is grown for about 24 additional hours. During this period of oleosynthesis, the single cell oil containing DHA is being synthesized and visible oil droplets become apparent. Those of skill in the art can readily calculate the time of fermentation required to achieve the expected amount of cell biomass based upon the added amount of YE. When that time has passed, the culture is grown for an additional 24 hours and harvested. In general the *C. cohnii* are cultivated for a time sufficient to produce single cell oil, usually from about 60 to about 90 hours, although this time is subject to variation.

From about 20 to 30% of the resultant biomass, using wild-type *C. cohnii*, comprises extractable oil. Strain selection can increase this percentage and such selection is within the scope of this invention. Preferably, the oil comprises greater than about 90% triglycerides having, in general, the following fatty acid composition.

- 15–20% myristic acid (C<sub>14:0</sub>)
- 20–25% palmitic acid (C<sub>16:0</sub>)
- 10–15% oleic acid (C<sub>18:1</sub>)
- 40–45% DHA (C<sub>22:6</sub>)
- 0–5% others

The crude oil is characterized by a yellow-orange color and is liquid at room temperature. Desirably, the oil contains at least about 20% DHA by weight and most preferably at least about 35% DHA by weight.

The organisms are harvested by conventional means, known to those of skill in the art, such as centrifugation, flocculation or filtration, and can be processed immediately or dried for future processing. In either event, the oil can be extracted readily with an effective amount of solvent. Suitable solvents can be determined by those of skill in the art. However, a preferred solvent is pure

hexane. A suitable ratio of hexane to dry biomass is about 4 liters of hexane per kilogram of dry biomass. The hexane preferably is mixed with the biomass in a stirred reaction vessel at a temperature of about 50° C. for about 2 hours. After mixing, the biomass is filtered and separated from the hexane containing the oil. The residual biomass, i.e. the single cell edible oil extracted biomass of the microorganisms, such as *C. cohnii*, can be used as an animal feed, containing as it does about 35–40% protein, 8–10% ash and 45–50% carbohydrates. The hexane then is removed from the oil by distillation techniques known to those of skill in the art. Conventional oilseed processing equipment is suitable to perform the filtering, separation and distillation. Additional processing steps, known to those of skill in the art, can be performed if required or desirable for a particular application. These steps also will be similar to those involved in conventional vegetable oil processing and do not comprise a part of this invention.

Isotopically labelled single cell oils, including labeled DHA, can be easily obtained in sufficient quantities to permit research into the metabolic pathways of DHA by the method of this invention. When <sup>13</sup>C-glucose or <sup>14</sup>C-glucose is provided as the reduced carbon substrate, labeled DHA results.

The present invention also includes food products, such as infant formulas and baby foods, as well as dietary supplements, which contain the single-cell oil containing DHA of the present invention. While those of skill in the art have recognized that infant formulas containing DHA are desirable, the prior art infant formulas contained DHA from fish oil, with its attendant unpleasant tastes and organoleptic characteristics. Furthermore, fish oil supplementation of infant formula includes the addition of eicosapentaenoic acid (EPA), an omega-3-fatty acid known to possess anticoagulant activity. Such an activity is not desirable in infant formula or baby food and the single cell oil described herein contains no significant quantity of EPA. Food products, such as infant formula, containing the single cell oil of the present invention do not have the unpleasant organoleptic characteristics of fish oil. The food products thus are more readily accepted by infants and adults alike. Preferably the infant formula of the present invention contains about 0.05% by weight of single cell oil containing DHA. The baby food of the present invention, having a more solid constitution, preferably contains about 0.5% by weight of single cell oil containing DHA. In both instances, most preferably, the oil contains at least about 35% DHA.

The present invention includes pharmaceutical products including single cell oil containing DHA. Preferably the products contain at least about 35% DHA. Exemplary of such pharmaceutical products is one suitable for use in providing parenteral nutrition to infants. Additionally, dietary supplements containing the single cell oil are encompassed. Preferably, such supplements are in the form of gelatin capsules encapsulating said oil.

The present invention also includes single cell oil containing DHA. Preferably the single cell oil contains at least about 20% by weight DHA. Most preferably the oil contains at least about 35% by weight DHA.

The present invention having been generally described, reference is had to the following non-limiting specific example.

## EXAMPLE

Into a 30-liter working volume STF was loaded a medium of one quarter strength artificial seawater. Six liters of IO were combined with 18 liters of tap water. The fermentor containing the medium was sterilized and cooled to 28° C. Four hundred ml of concentrated YE (455 g/l), 900 ml of glucose syrup (400 g/l) and one liter of inoculum from a seed fermentor containing about  $2 \times 10^7$  cells/ml or a biomass of 20 g/liter (yielding a final concentration of about  $10^5$  cells/ml or a biomass of about 700 mg/liter), were added to the medium. Agitation was set at 120 cm/sec tip speed and aeration was set at 1 VVM (30 liters per minute). Additional glucose syrup (900 ml) was added after 30 hours and another 4.2 liters over the next 42 hours. Thus 6 liters of glucose syrup were added in total. Concentrated YE solution (400 ml) was added at hour 6 and another 1.2 liters were added over the next 48 hours until a total of 2.0 liters had been added. To maintain the D.O. at greater than 20%, at 24 hours the agitation tip speed was increased to 150 cm/sec and at 48 hours to 160 cm/sec. At 72 hours, the tip speed was increased to 200 cm/sec and the culture was permitted to grow for an additional time sufficient to convert the final charge of glucose into cellular oil. The culture was then harvested by centrifugation with the cell pellet retained. The harvested pellet of cells was frozen and dried (lyophilized) to about a 4% moisture content. Hexane (2.8 liters) was added to the dried biomass and stirred in a glass kettle for 1.5 hours at 50° C. A rotary evaporator was used to remove the hexane, producing about 175 g of crude DHA-containing oil.

We claim:

1. A method of producing a single cell edible oil containing at least about 20% docosahexaenoic acid (DHA) in triglyceride form comprising:

cultivating heterotrophic microalgae of the class Dinophyceae capable of producing said single cell oil in an aerated fermentor containing a nutrient solution having a limiting nitrogen source and an oxygen level of at least about 10% of air saturation level and continuing cultivation to achieve a cell density of at least about 10 grams biomass per liter of nutrient solution,

wherein the concentration of the nitrogen source in the nutrient solution is limited sufficiently to induce said microalgae to produce the single cell oil at a concentration of at least about 2 grams per liter of nutrient solution, and

recovering said single cell oil.

2. The method of claim 1, wherein the microalgae is of the genus *Cryptocodinium*.

3. The method of claim 2, wherein said microalgae comprises *Cryptocodinium cohnii*.

4. The method of claim 3, wherein said nutrient solution comprises seawater.

5. The method of claim 4, wherein said seawater comprises an artificial seawater.

6. The method of claim 5, wherein said nutrient solution comprises a one-quarter strength solution of artificial seawater.

7. The method of claim 4, wherein said nutrient solution further comprises a reduced carbon source.

8. The method of claim 7, wherein said reduced carbon source comprises glucose.

9. The method of claim 4, wherein said limiting nutrient comprises an organic nitrogen source.

10. The method of claim 9, wherein said nitrogen source comprises yeast extract.

11. The process of claim 10, wherein the ratio of said glucose to said yeast extract is from about 2 to 4 parts glucose to 1 part yeast extract.

12. The method of claim 11, further comprising maintaining a dissolved oxygen content at a range of from about 10% to about 50% of air saturation.

13. The method of claim 12, wherein said range is from about 20-30%.

14. The method of claim 13, wherein said fermentor is a stirred tank fermentor having a turbine.

15. The method of claim 14, wherein said turbine agitates said nutrient solution, thereby providing oxygen to said microalgae.

16. The method of claim 1, wherein said aerated fermentor is aerated at a rate of 1 volume of air per volume of fermentor per minute.

17. The method of claim 1, wherein said nutrient solution comprises  $^{13}\text{C}$ -glucose or  $^{14}\text{C}$ -glucose.

18. The method of claim 1, wherein the microalgae is cultivated to a density of at least about 20 g/liter of nutrient solution.

19. A method for the production of a single-cell-edible oil containing at least 20% DHA in triglyceride form and lacking a fishy taste comprising:

a) adding to a fermentor initially containing a nutrient solution comprising about one-quarter strength artificial seawater, 1-2% glucose and 0.4-0.8% yeast extract, about 0.5-1.0 g dry wt/liter (about  $10^5$  cells/ml) of *C. cohnii*,

b) cultivating said *C. cohnii* at a temperature of from about 15° C. to about 34° C. and a pH of from about 5.0 to 9.0,

c) incrementally adding glucose and yeast extract to said nutrient solution for about 56 hours,

d) adding additional glucose to said nutrient solution for about 16 additional hours to induce said *C. cohnii* to produce a single-cell edible oil,

e) maintaining a dissolved oxygen content of about 20-30% of air saturation level throughout said cultivation,

f) harvesting said *C. cohnii* after about 60 to 90 hours, and

g) recovering said single-cell edible oil.

20. The process of claim 19, further comprising extracting said single cell edible oil from said *C. cohnii*.

21. The process of claim 20, wherein said extraction is by treatment with a solvent such that said oil is extracted into said solvent.

22. The process of claim 21, wherein said solvent comprises hexane.

23. The process of claim 22, wherein said hexane is applied to said *C. cohnii* at a rate of about 4 liters per kilogram of dry biomass of said *C. cohnii* and is mixed with said biomass in a stirred reaction vessel for about 2 hours at about 50° C.

24. The process of claim 23, further comprising filtering said hexane containing extracted oil from said biomass and then removing said hexane by distillation from said oil.

25. A single cell-edible oil comprising at least 20% docosahexaenoic acid in triglyceride form, said oil produced according to the method of claim 1 or the method of claim 19.

\* \* \* \* \*



**RELATED PROCEEDINGS APPENDIX**

None.